An analysis of quenching and extraction techniques for performing metabolomics in *Acidithiobacillus ferrooxidans*

by

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Abstract

I present here a protocol for accurate liquid chromatography – mass spectrometry based determination of the metabolome of *Acidithiobacillus ferrooxidans*. *At. ferrooxidans* metabolic processes contribute to the environmentally destructive process of acid mine drainage (AMD), and metabolomic profiling can offer insight into how *At. ferrooxidans* contributes to these processes. Metabolism must be stopped (quenched) instantaneously while limiting the amount of cell damage. I tested a wide variety of quenching solutions and found that ammonium formate was the most effective quenching solution. An accurate metabolomics analysis also requires extraction of as large a number of metabolites as possible, with as little chemical bias (in extraction or degradation) as possible. I tested a wide variety of extraction solutions and found that isopropanol:methanol:water was the best extraction solution. Standardizing a protocol to quench and extract metabolites in *At. ferrooxidans* will contribute to understanding how *At. ferrooxidans* contributes to the AMD phenomenon.

Keywords

Acidithiobacillus ferrooxidans, Acid Mine Drainage, Metabolomics, Quenching, Extraction, Leakage, Cold Shock Phenomenon, Tandem Mass Spectrometry

Co-Authorship Statement

Chapter 1 is an introduction to acid mine drainage, the effects of *Acidithiobacillus ferrooxidans*, its importance to environmental damage and the process of studying this bacterium through metabolomics. Chapter 2 explains the methodology and results obtained through the described protocols. Chapter 3 is a future directions section where and how this protocol can be applied. Thomas J.S. Merritt (TJSM), Nadia C.S. Mykytczuk (NCSM) and Arielle K. Bieniek (AKB) provided editorial and organizational feedback to all three chapters.

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List of Abbreviations

AAW – Acidic acetonitrile water
AC – Alternating currents
ADP – Adenosine diphosphate
AMD – Acid mine drainage
AMP – Adenosine monophosphate
AQ – After quenching
ATCC – American type culture collection
ATP – Adenosine triphosphate
BA – before analysis
^{13}C – carbon isotope with 6 protons and 7 neutrons
CMW – chloroform:methanol:water solution
CS – Cell supernatant
DC – direct current
ES – Extraction solution
ESI – Electrospray Ionization
FMF – Find molecular features
GC – Gas chromatography
HILIC – Hydrophobic interaction chromatography
I – Quenching method
IMW – Isopropanol:methanol:water solution
kDa – Kilodalton
L – Liter
LC – Liquid chromatography
M/Z – Mass-charge ratio
MGCDW – Mass gram cell dry weight
MS – Mass spectrometry
N – Quantifiable value for leakage or intracellular metabolites
NMR – Nuclear magnetic resonance
PCA – Principal component analysis
Qq-TOF – Quadrupole time of flight
QS – Quenching solution
T – Time
TOF – Time of flight
µL – Microliter
µm – Micrometer
V/V – Volume per volume
W/V – Weight per volume
X – Indicated metabolite

Chapter 1 Introduction

1.1 Overview

Metabolomics is the study of small molecules found within an organism's tissue or sample (Fiehn *et al*., 2001; Oliver *et al*., 1998; Tweeddale *et al*., 1998;). Metabolomics is an emerging science with powerful high throughput analytical techniques still in development (Nicholson & Lindon, 2008), including development of protocols for a variety of different sample types. Performing metabolomics on microbial samples poses a unique challenge in that the microbes need to be removed from their liquid environment for analysis, but need to be kept in that medium to prevent environmental stress from altering their metabolome. Microbial metabolism needs to be instantaneously stopped while the microbes are still in their liquid environment, without damaging their cell membrane, and then separated from that media as quickly as possible (de koning and van Dam, 1992; Schaefer *et al*., 1999). In addition, the metabolites within a cell need to be extracted from the cell without biasing, excluding, or degrading any metabolite pools. Owing to the vast diversity of microbial membranes, cell wall structures, and cytosolic matrices the appropriate protocol for stopping a cell's metabolism and extracting metabolites with the greatest efficiency must be determined on species by species basis (Bolten and Wittman, 2008; Shin *et al*., 2010).

My MSc thesis involved developing an extraction protocol for *Acidithiobacillus ferrooxidans* a microbial species found in Acid Mine Drainage (AMD). *At. ferrooxidans* is an organism of interest because it is a major contributor to the production of AMD; it is a chemolithotroph that can use ferrous iron (as well as other metals) as an electron donor producing ferric iron that is a substrate in overall chemical reaction that results in AMD

production (Singer and Stumm, 1970). AMD is a major global environmental problem and developing a protocol to study the metabolome of *At. ferrooxidans* can give us insight into the biology that causes this problem as well as allowing us to ask fundamental questions about chemolithotrophy.

1.2 Acid mine drainage and Acidithiobacillus ferrooxidans

AMD is highly acidic, metal rich, water that drains from mining disturbed areas. The acidic nature of AMD is the result of the oxidization of metal sulphides in mining waste. AMD can be produced abiotically, but microorganism metabolism accelerates iron oxidization by up to six orders of magnitude (Edwards *et al*., 1999; Singer and Stumm 1970;). Acid mine drainage sites are found globally. In Canada, strong environmental regulations restrict AMD sites largely within controlled tailings ponds, impoundments designed to contain draining water and control its discharge. Tailing ponds contain microbial communities that are taxonomically diverse but functionally stable (Strenten-Joyce *et al*., 2013; Tan *et al*., 2009). The organisms that accelerate the production of AMD are iron and sulfur oxidizing organisms, with the specific iron oxidizers present in an AMD site corresponding to the pH and other physiochemical properties of the site (Kuang *et al*., 2013; Méndez-Garcıa *et al*., 2014; Mueller *et al*., 2011; Tan *et al*., 2009). *Acidithiobacillus ferrooxidans* is the predominant iron oxidizing organism at a pH of 1.5-2.4, whereas *Leptospirillum ferrooxidans* is dominant at slightly lower pH's and the archaeal *Ferroplasma* genus is thought to be the dominant iron oxidizer at pH's between 0-1.7 (Bond *et al*., 2000; Dopson *et al*., 2004; Edwards *et al*., 1999; Schrenk *et al*., 1998).

1.3 Local Relevance of AMD and At. ferrooxidans

AMD is especially relevant to Sudbury, Ontario Canada, because mining has historically been the dominant industry in the area, resulting in numerous tailing ponds near the city. The Vale Copper Cliff tailings site is one such site, and has previously been studied using bioinformatics approaches to determine the composition of its microbial community. The research showed that *At. ferrooxidans* is the dominant member of said community (Auld *et al*., 2013; Leduc *et al*., 1993).

Because of the central role of *At. ferrooxidans* metabolism in the production of AMD (Singer and Stumm, 1970), studying the metabolome of *At. ferrooxidans* will contribute to the understanding of the processes contributing to AMD.

1.4 Metabolomics

Metabolomics is the study of the set of metabolites within an organism, tissue, or sample. Metabolites are defined as chemicals that are smaller than a thousand Daltons, and include sugars, cell signaling molecules, amino acids and antibiotics (Fiehn, 2001; Oliver *et al*., 1998; Tweeddale *et al*., 1998). The set of metabolites in a cell form a large network of chemical reactions in which the output of one enzymatic reaction is the input for another. In comparison with genomics, transcriptomics or proteomics, metabolomics is still very much in its infancy, due, at least in part, to the complex and diverse chemical nature of the metabolome and the difficulty in identifying and quantifying chemically diverse metabolites (Dettmer *et al*., 2007; Kamleh *et al*., 2008; t'Kindt *et al*., 2010). Metabolomics is defined by diversity and the complex nature of the metabolome that consists of thousands of chemically distinct molecules. In contrast, the proteome includes only twenty distinct amino acids (although the diversity of proteins is

enormous), while in genomics and transcriptomics, chemical diversity is limited to different combinations of four nucleotides. Attempting to identify all the components of the metabolome is formidable task requiring the use of several analytical instruments.

1.5 Analytical Analysis of the Metabolome

Analytical chemistry is the identification, separation, and quantification of matter. In the early 2000's desktop analytical techniques became widely available and the field of metabolomics began to gain traction. There are many different analytical technologies that can be utilized for metabolomics, each with different characteristics that make them more or less suited to microbial applications. Common analytical techniques that can be applied to metabolomics include hyphenated mass spectrometry (-MS) techniques and nuclear magnetic resonance (NMR) (Fiehn, 2001; Gamache *et al*., 2004). Nuclear magnetic resonance is used to elucidate molecular structural information, which can be used to identify unknown molecules. However, NMR has a high detection limit meaning it isn't sensitive enough to detect compounds present in small quantities, and is unable to differentiate between compounds that are analyzed as part of a complex mixture (Wishart, 2008). The latter characteristics make it an impractical technology to be applied to a microbial metabolomic analysis, which involves the analysis of complex mixtures of metabolites present at low concentrations. Conversely, mass spectrometry can be used to differentiate between compounds being introduced simultaneously and is more sensitive (Dettmer *et al*., 2007). Tandem MS can be used to identify unknown compounds. Tandem mass spectrometry is a technique in which ions of a single mass (parent ions) are isolated and fragmented to produce reproducible fragmentation patterns (daughter ions) that can be compared to the fragmentation patterns of known chemicals

(Dettmer *et al*., 2007). The drawback to tandem MS chemical identification is that one must be able to match MS fragmentation patterns to known fragmentation patterns that are present in metabolomic databases. While current metabolomic databases contain the fragmentation patterns of a variety of metabolites, they are known to not completely cover all possible metabolites and not all the metabolites present in these databases have the tandem MS spectra, indicating that many chemicals can be detected but not named (Kind *et al*., 2009). Overall, MS techniques are more appropriate than NMR to use for microbial metabolomics applications (Pan *et al*., 2007) however there are many variations in MS technology that alter its applicability to microbial metabolomics (Baidoo *et al*., 2012). The variations to MS techniques include altering ionization methods, different mass analyzers, as well as the potential for including front end separation techniques.

1.6 Ionizing Small Molecules

MS is performed by transforming a chemical into its gaseous phase and then ionizing it. Chemical ionization occurs at the ion source, via one of two methods, soft or hard ionization. Hard ionization occurs when a large amount of energy is transmitted to a molecule causing the molecule to fragment into what is known as daughter ions. The daughter ions have a lower mass to charge ratio than the parent ion did. Soft ionization ionizes molecules by transmitting a low amount of energy to a molecule, this prevents fragmentation, so the molecular ion is always the ion measured. Soft ionization is the best technique to use when analyzing small molecules (metabolites) that would be fragmented into non-descript pieces via a hard ionization technique (Dettmer *et al*., 2007; Lee *et al*., 2010).

1.7 Electrospray Ionization (ESI) and Matrix Effects

Many different soft ionization techniques exist, but the most common technique is electrospray ionization (ESI) (reviewed in: Garcia *et al*., 2008; van der Werf *et al*., 2007). ESI has the broadest coverage of all ionization techniques making it well suited to an untargeted metabolomics analysis (Wilm, 2011). One main disadvantage of ESI techniques is its susceptibility to matrix effects. The term "matrix effects" refers to the phenomenon in which many ions exit the ESI source simultaneously. Matrix effects can affect the ability of a given metabolite to ionize, and can lead to ion suppression or enhancement of the metabolite which results in under- or over-estimation of the amount of a chemical(s) that are present (Mueller *et al*., 2011). Ion suppression and enhancement can be especially problematic when working in microbial metabolomics due to the fact that microbes are grown in "salty" media and salts are known to cause ion suppression and enhancement (Olivares *et al*., 1986). In addition, microbial cultures contain phospholipids that are known to cause ion suppression (Ismaiel *et al*., 2008). Ion suppression can be greatly reduced by coupling MS to a front end separation technique such as gas or liquid chromatography to reduce the number of different metabolites that are entering the ionization chamber at one time, these are discussed in more detail later in the chapter.

1.8 Mass Analyzers for Metabolomics Research

After ionization, ions are moved through an electromagnetic field of known strength, and the mass-charge (m/z) ratio of the ions determines their velocity. The component of the MS that determines the m/z ratio, and moves the ions, is called the mass analyzer. Different mass spectrometers contain different types of mass analyzers

such as quadrupoles, hexapoles, ion traps, and time of flight chambers (TOF) (De Hoffman, 2005; Reviewed in: El-Aneed *et al*., 2009). Using a combination of more than one type of mass analyzer is common practice in metabolomics laboratories, as combining different techniques provides more versatility in applications, and can increase the efficiency of said applications (Reviewed in: El-aneed *et al*., 2009). In my MSc research I used a triple quadrupole time of flight mass spectrometer (Qq-TOF).

1.9 Quadrupoles

A quadrupole is a multipole mass spectrometer that consist of four rods connected to each other electrically, arranged in parallel, in a cylindrical formation. The ions travel through the cylinder where they can be manipulated through the use of direct currents (DC) or alternating currents (AC). If only a DC is applied to the ions, then the quadrupole acts as a guide to move the ions through the multipole. However, if an AC radio frequency current is applied then ions will have an oscillating path, and only ions with a certain m/z ratio will reach the detector, while all other ion sizes will have an unstable trajectory and crash into the rods (Dawson, 1986; De Hoffman, 2005). The application of AC conditions can, then, be used to select specific ions, i.e. specific metabolites. A quadrupole allows for both full scan mode, in which all ions are measured based on their m/z ratio; and selected ion monitoring, where only ions of a specific m/z ratio are allowed to reach the detector. To increase the usefulness of a quadrupole, oftentimes triple quadrupole configurations are used in which three quadrupoles are arranged in a linear fashion. The first quadrupole is used to filter masses or to scan all of them. The second quadrupole is used as a collision cell in tandem mass spectrometry applications. In the collision cell, daughter ions will be produced, and the third

quadrupole either scans all of the ions or only sends specific ones to the detector (De Hoffman, 2005; Reviewed in: El-aneed *et al*., 2009).

1.10 Time of Flight (TOF)

A TOF mass spectrometer uses voltage to accelerate an ion down a flight tube, and the time it takes for the ion to reach the detector is inversely proportional to its mass-to-charge ratio. A longer flight path is generally correlated with a more accurate instrument and to increase the precision and resolution of a TOF, as well as correct for the kinetic energy distribution caused by the initial acceleration (addition of kinetic energy), a reflectron can be used to increase the flight path. A reflectron consists of a uniform electric field that is perpendicular to the flight path of the ions. Ions with more kinetic energy will penetrate the reflectron more deeply thereby increasing their flight path, ions with the same mass-to-charge ratio but less kinetic energy penetrate less deeply into the reflectron and thereby have a shorter path to the detector. Inclusion of a reflectron standardizes experiments for molecules with the same mass (De Hoffmann, 2005; Reviewed in: El-aneed *et al*., 2009).

1.11 QqTOF

In a QqTOF mass spectrometer, the third quadrupole has been replaced with a TOF, any fragmentation or mass selection steps occur in the first two quadrupoles, while the TOF is used to record the spectra. The instrument benefits from high resolution and mass accuracy over a large range of masses. In addition, it has the ability to perform tandem mass spectrometry, and select from specific ions either in tandem or regular mass spectrometry applications (Chernushevich *et al*., 2001; Verhoeven *et al*., 2006).

1.12 Chromatographic Separations for Metabolomics

As previously mentioned, chromatographic separation prior to ionization can greatly reduce the matrix effects that ESI is susceptible to. Gas chromatography (GC) and liquid chromatography (LC) are commonly used methods of chromatographic separation. For a metabolomics analysis, LC allows for broader coverage of the metabolome because GC requires analytes be volatile for analysis and not all metabolites are volatile. Metabolites can be made volatile through with complicated derivatization procedures that transform a chemical into a more volatile derivative. Oftentimes two dervitization procedures are needed such as methoximation followed by silylation (Gullberg *et al*., 2004; Koek *et al*., 2011; Schauer *et al*., 2005; Smilde *et al*., 2005). To allow volatilization, you can modify chemicals through derivatization, but this process can be lengthy and difficult and still not give as broad a metabolite coverage as LC. LC works by passing analytes in a liquid mobile phase through a column with a stationary phase. The analytes undergo a pseudo equilibrium distribution between the mobile and stationary phase, with the amount of time the metabolites in the mobile phase spend in association with stationary phase determining how long it takes for them to elute. The overall separation of a mixture depends on the chemical nature of the various constituents. Metabolite separation is determined by retention time which can be altered by modifying the mobile phase, e.g. changing the pH, or less commonly, by changing the temperature of the column. Retention time and metabolite separation can be greatly modified through the use of different column stationary phases, and the choice of column type is, therefore, an important step in LC-MS metabolomics.

1.13 Hydrophilic Interaction Chromotography (HILIC)

The type of column used determines which analytes can be retained and resolved. HILIC (Hydrophilic Interaction Chromatography) is a common separation technique used for metabolomic analyses because it is relatively good at separating polar compounds (reviewed in: Buzewski and Noga, 2012). Metabolic processes generally result in the addition of polar groups to a molecule in order to promote elimination from cellular tissue, therefore many metabolites can be resolved on a HILIC column. HILIC works by using a polar stationary phase with a highly organic mobile phase that contains a small amount of water which forms a layer on top of the stationary phase. Polar analytes are retained in this layer of water through a variety of interactions including electrostatic interactions, hydrophilic partitioning of an analyte (between aqueous phase and mobile phase), and even van der Waals interactions (that can be present when very low organic solvent is present). HILIC is well suited for metabolomics analyses, but does have some disadvantages: HILIC requires a long equilibration time due to the need for the column to be "wetted" and it cannot resolve structural isomers (Bajad *et al*., 2006; Buzewski and Noga, 2012; Luo *et al*., 2007). Although HILIC does have some disadvantages it is still well suited for a metabolomics analysis due to its ability to resolve highly polar compounds. In addition, when HILIC is coupled to an ESI technique it can offer increased sensitivity over a reverse phase (non polar stationary phase with polar mobile phase) technique due to the highly organic mobile phase being used. The highly organic solvent is more volatile than an aqueous solvent, which increases desolvation, the removal of a solvent from a metabolite (Grumbach *et al*., 2004). Increased desolvation improves the efficiency of solvent removal from a metabolite and lowers detection limits

(makes it possible to detect metabolites at lower concentrations). To summarize using a HILIC column with a Qq-TOF is a good approach for microbial metabolomics because it allows us to separate and resolve low concentration polar analytes in a repeatable way and also gives us the capability of identifying unknowns through the tandem mass spectrometry.

1.14 Microbial Metabolomics

There are two ways of applying a hyphenated mass spectrometry technique to microbial metabolomics: metabolic footprinting, and metabolic fingerprinting. Metabolic footprinting is a whole broth sampling technique that consists of determining the metabolite content of a microbe, as well as the environment it was cultured in. In theory, metabolic footprinting allows one to see both the endo- (intracellular) and exo-metabolome (extracellular). In reality, the endometabolome is often hidden by the greater volume of the exometabolome (Oliver *et al*., 1998; Tweeddale *et al*., 1998; Fiehn *et al*., 2001). The volume of cell supernatant (medium) is many times greater than the biovolume of a cell such that the exometabolome often contains such an abundance of metabolites that it surpasses the endometabolome, and as such masks the metabolic contributions of the latter (Paczia *et al*., 2012). In addition, there are technical issues associated with looking at a metabolic footprint especially when using LC-ESI-MS. The main issue is that the media a cell is grown in contains high concentrations of salts which can lead to ion suppression and enhancement, making quantification difficult. In addition, injecting such a "dirty" solution increases background noise making it difficult to identify low intensity metabolites. Furthermore, the media has a tendency to clog a column quickly which can cause increases in pressure that lead to shifts in retention time

as well as bad peak shape (Taylor, 2005). To summarize it is difficult to perform metabolic footprint for both biological and technical reasons, in order to accurately observe the endometabolome of the cell, metabolic fingerprinting is required.

Metabolic fingerprinting is achieved when a microbe is separated from its environment in order to examine the metabolic activity occurring inside the cell. Prior to this separation step the metabolism needs to be stopped, or quenched as it is commonly referred to. At the same time, it is crucial that when metabolism is quenched, the cell membrane is left intact so that metabolites do not leak from the cell, which would confound the endo- and exo-metabolomes. In this study metabolic fingerprinting will be used as we are interested in the metabolism of the actual living organism, and not just the compounds it is excreting into the environment (Fiehn, 2001).

1.15 Metabolic Quenching

Metabolic quenching essentially allows us to arrest the metabolism of an organism at a moment in time. Regardless of what taxonomic group is being studied, the most common way of arresting the metabolism is literally through freezing the organism or tissue sample in liquid nitrogen, although this technique is much more commonly seen in animal and plant cell metabolomics than in microbial metabolomics. In microbial metabolomics, we see a higher diversity of metabolic quenching methods being used including exposure to either high or low temperature or pH extremes.

In microbial metabolomics, exposure to extremely low pH has been used to arrest cellular metabolism. Perchloric acid is the most commonly used acid and it has been applied to a variety of microbes including *E. coli* and *Saccharomyces* (Klingenber and Pfaff, 1967; Koek *et al*., 2006). Perchloric acid quenching was not considered an

appropriate quenching mechanism for working with *At. ferrooxidans,* because extreme acidic pH's are the norm for this organism, and would not be sufficient to quench metabolic activity. A rarer, but still implemented, method of arresting enzymatic activity consists of exposure to high temperature generally using boiling ethanol solutions (Gonzalez *et al*., 1997). This method was not considered a suitable quenching solution for *At. ferrooxidans* because of the potential for degradation of heat sensitive metabolites. Furthermore, although heat does effectively denature proteins, there is the potential for renaturation after heat is removed (Gonzalez *et al*., 1997; Marharjan *et al*., 2003;Winder *et al*., 2008).

The most popular type of microbial quenching is a cold methanol based method, which was originally developed for studies of yeast but has frequently been applied to in studies of a variety of microbes including bacteria and fungi (de koning and van Dam, 1992). Although this method has successfully been applied to many different species of microbe, it can be a non-ideal quenching mechanism due to the observed metabolite leakage that can be attributed to the "cold shock phenomenon" (Wittmann *et al*., 2004).

1.16 Cold Shock Phenomenon and Metabolite Leakage

The cold shock phenomenon is the loss of membrane integrity when the cell membrane is exposed to a cold organic solution (Wittmann *et al*., 2004), causing metabolites to leak out of a cell. Cold shock phenomenon effects different cell types to varying degrees. Eukaryotic cells are effected the least by cold shock phenomenon and metabolite leakage, Gram positive bacteria are intermediate in their sensitivity, and Gram negative bacteria are the most sensitive based on their membrane composition (Bolten *et al*., 2008; Wittmann *et al*., 2004;). Although metabolite leakage can never be fully

stopped in prokaryotic cells, it can be minimized in many species specific ways. These include the application of buffering agents at specific ionic strengths to prevent osmotic stress, adjusting the pH of a quenching solution to match the pH of the media an organism is grown in, and potentially using glycerol because of its cryoprotectants capabilities (Faijes *et al*., 2007; Spura *et al*., 2009; Villas-Bôas & Bruheim., 2007). The effect of ionic strength on the ability of a quenching solution to limit metabolite leakage has been tested on a variety of prokaryotes including *Corynebactrium glutamicum* and *E. coli* and an ionic strength of 0.8-0.9% w/v is generally appropriate for bacteria that are not halophilic (reviewed in: Xu *et al*., 2014). In addition, a variety of buffering agents have selected for use including, buffers that are known to work well with a specific bacteria (i.e. the use of HEPES buffer with *E.coli*; Hoque *et al*., 2005; Oldiges *et al*., 2004), buffers that are being used in the liquid chromatography method (i.e. the ammonium carbonate with a HILIC gradient where the aqueous phase contains 10mmol ammonium carbonate; Faijes *et al*., 2007) and finally generic salts that can be compatible with a large amount of bacteria (i.e. physiological saline; Spura *et al*., 2009; Xu *et al*., 2014). A few studies have included glycerol, or glycerol-saline, to replace the aqueous phase of 60% cold methanol. The main disadvantage of this method is that glycerol sticks to the cells and produces a larger glycerol peak that masks many of the metabolites in a GC-MS metabolic profile (Villas-Bôas & Bruheim, 2007). In LC-MS it is possible to separate out glycerol although glycerol itself is a metabolite and using it as cryoprotectant gets rid of the ability to detect it in a cell's metabolome (Villas-Bôas & Bruheim, 2007). In general, although glycerol can work as a cryoprotectant the use of it limits the

versatility of a protocol as it can no longer be used with a GC-MS (Spura *et al*., 2009; Villas-Bôas & Bruheim., 2007).

For my thesis, I tested three quenching solutions that are based on cold methanol quenching. All the extraction solutions used were 60% methanol with 40% aqueous at a pH of 2.5. The solvent percentages were choosen because they are predominately used in microbial metabolomics protocols (Canelas *et al*., 2008; Link *et al*., 2008; Winder *et al*., 2008). The pH was set by the optimal growth media for *At. ferrooxidans*, to minimize any stress and limit prevent metabolite leakage. In addition, the aqueous portion contains 0.8-0.9% w/v of salt, a relatively low salt concentration because these bacteria are not halophilic. I tested three different salts to determine their ability to limit leakage: ammonium formate because it is used as a buffer in our mobile phase of liquid chromatography, physiological saline because of wide applicability, and a modified version of TK salts which are the salts present in the microbes' normal growth media.

It is necessary to quantify the amount of leakage that occurs in order to understand and properly correct for biases. Quantification of metabolite leakage can be done by comparing the amount of metabolites in the cell supernatant before quenching, to the amount of metabolites in the cell supernatant immediately after quenching (Tillack *et al*., 2012).

1.17 Metabolite Extraction

In order to ensure sample representativeness, metabolites must be extracted immediately following the quenching of cellular metabolism. The first step in metabolite extraction is to disrupt the cell membrane through the use of mechanical disruption, chemical disruption, or both (Knee *et al*., 2013; Smart *et al*., 2010). The most common

methods of mechanical disruption include sonication, vigorous vortexing and freeze-thaw cycle(s). These types of mechanical disruption are seen throughout the literature, and have been applied to a variety of organisms and tissue types, not just microbes (Martinez *et al*., 2013; Smart *et al*., 2010; Zivkovic *at al*., 2009). Although sonication has been used in many protocols in the past, it is generally frowned upon due to the fact that that it will break down cellular organelles. Cellular organelles are large macromolecular structures that can be hydrolyzed during sonication into smaller chemical components (less than 1 kDa), which will then be falsely recognized as metabolites through analytical analysis (Clark and Christopher 2000; Ershov 1998; Wu *et al*., 2008). This disruption results in the production of chemical artifacts that can cause the overestimation of the quantity of metabolites inside a cell. In contrast, bead-beating, freeze-thaw cycles, and vigorous vortexing are much gentler form of mechanical digestion, which do not cause the same formation of chemical artifacts. Bead beating is most frequently seen in conjunction with insect tissues (Knee *et al*., 2013). Prokaryotic microbes are most frequently extracted through freeze thaw cycles and vigorous vortexing in conjunction with chemical permeabilization (Smart *et al*., 2010; Zivkovic *et al*., 2009;). The chemicals used to permeabilize the cell also serve as the extraction solvents. A variety of extraction solvents have been used regardless of sample type, ideally an extraction should be non-toxic, non-selective and have a high solubilizing power. In addition, it is necessary that an extraction solvent be compatible with the analytical technology of choice and must be suitable for the specific sample matrices. As microbes have unique cytosolic matrices there is no universal extraction protocol (Mushtaq *et al*., 2014).

Overall, the most popular extraction solvents are organic alcohols and water. Different combinations of methanol, ethanol, isopropanol and water are commonly seen in a variety of metabolomics literature, and are oftentimes used for broad based plant extractions (Chauvin *et al*., 2013; Grata *et al*., 2008). Organic alcohols and water have also been used extensively in microbial metabolomics. In work done on *Sarcophagus degradans*, a Gram negative bacterium, it was shown that either acetonitrile/methanol/water or isopropanol/methanol/water were the best extractions solvents for a broad based extraction as compared to pure methanol and an acetonitrile water mixture (Shin *et al*., 2010). Conversely, others have concluded that acidic acetonitrile and water were the best extraction solvents to use in *E.coli* metabolomics because that extraction solvent allowed for a both a broad based extraction as well prevented the degradation of triphosphates that was seen in methanol/water extractions (Rabinowitz and Kimball *et al*., 2007).

Chloroform/methanol mixtures are also used as extraction solutions, however they have been shown to perform poorly with polar metabolites and is sometimes avoided due to its toxicity (Zaldivar *et al*., 2002). The addition of water to the chloroform/methanol extraction can partially remedy this problem and this combination is a common extraction solvent in *Drosophila* metabolomics (e.g. Knee *et al*., 2013). Furthermore, chloroform/methanol/water is considered the ideal extraction solvent for analyzing the metabolome of *Chlamydomonas reinhardtii*, a eukaryotic microbe (Bolling and Fiehn *et al*., 2005).

For this study we have tested out three different extraction solutions, the first of which was a chloroform/methanol/ water extraction. This extraction has worked well in a

variety of studies such as metabolomics research in *Drosophila* (Knee *et al*., 2013), so we know it is compatible with our analytical technology and can extract many of the metabolites that we can name. The second extraction solution I have tested is a acidic acetonitrile/water extraction solution. This is because we have had trouble in the past identifying many triphosphate compounds using the chloroform/methanol/water extractions and this will allow us to test if we can remedy that problem while still performing a broad based analysis. Finally, we have tested an isopropanol/methanol/water extraction as organic alcohols mixed with water have consistently performed well in the microbial metabolomics experiments.

To limit wear and tear on the instrumentation and improve metabolite resolution and identification, the extracted solution needs to be as clean as possible of cellular debris prior to analysis. This requirement poses a paradox: how do we clean a solution without losing metabolites? Ideally a solution should be as clean as possible in order to eliminate the matrix effects that plague ESI sourced analytical applications in order obtain the best sensitivity possible. However, there is also a need to retain all of the metabolites within a solution (Annesley, 2003). The most popular methods to clean up solutions include centrifugation, the use of size exclusion filters, as well as evaporation, and many of these methods can be used in tandem. Size exclusion filtration uses a filter that has a molecular weight cutoff point of 3-5 kDa, which effectively eliminates most of the large proteins that can cause contamination in an LC-MS. The use of size exclusion filtration is disadvantageous as use of filters can cause metabolite biases for certain compounds since groups of metabolites have an affinity for size exclusion filters (Elhamili *et al*., 2011). In

order to avoid these problems, we will only use centrifugation to exclude large particulate matter.

1.18 Protocol Validation

To date, no "universal" protocol for microbial metabolomics has been developed and given the diversity of microbes it is possible there will never be one. As a result it is currently convention to validate a protocol for a microbe if there isn't one already available in the literature. Protocol validations focus on measuring the amount of metabolite leakage caused by different quenching solutions, the number of metabolites a solution can extract as well as the recovery value for specific metabolites.

Metabolite leakage is a function of membrane and cell wall composition and, as each microbe has a unique membrane and cell wall leakage, will be organism and metabolite specific. In order to determine how much leakage occurs, and which compounds are leaking, one can first take a sample of the cell supernatant and determine what metabolites are normally found in it. The second step is to take an aliquot of cell supernatant after quenching and determine how many metabolites are now found in the quenching solution, the addition of any new metabolites is due to metabolite leakage. This step can be done using equation 1.

1. $N_{leakage}=NQS-NCS$

Where "N" indicates a quantifiable value for leakage, and "QS" and "CS" indicate quenching solution and cell supernatant respectively. This equation is used to measure the amount of cell leakage (Canelas *et al*., 2009).

In addition, the fact that each microbe has a unique cytosolic matrix further compounds the need for a microbe, and metabolite-specific extraction protocol. No

extraction solution has been found that is able to completely extract all metabolites. We can, however, determine which extraction solutions give the "best" overall results. What solution performs "best" can be very specific (e.g. in studies targeted at specific metabolites), but is often, simply, what solution recovers the greatest number of different metabolites with the least amount of degradation. In order to determine which solution meets this criterion, one must measure how many metabolites are extracted and how well they are recovered. The first measurement is done by just determining the number of metabolites that show up after extraction. The second measurement is done to see how completely these metabolites are extracted and recovered. The second measurement is performed by adding a variety of ^{13}C labelled standards immediately after quenching, as well as immediately prior to analysis (one sample is spilt in two). This protocol allows us to calculate how much of the individual compound is recovered, and also allows us to take into account matrix effects by comparing the level of ^{13}C labelled standards that are present at 100% in the sample where they were added immediately before analysis, to the level of ^{13}C labelled standards that were added before extraction (Birkmeyer *et al*., 2005). This measurement is performed using equation 2.

2. $Recovery_{x,i} = [x]_{iAQ} / [x]_{iBA}$

Where "x" indicated metabolite, "i" stands for quenching method and the upper indexes BA and AQ stand for before analysis and after quenching (Canelas *et al*., 2009). Overall understanding the recovery of specific metabolites gives a better understanding of how specific extraction solutions can bias our results, which will allow us to make more accurate conclusions.

1.19 Conclusion and Thesis Objectives

To conclude, the metabolome of *At. ferrooxidans* is a point of scientific and industrial interest due to its role in the AMD environment. In order to ask any questions about the metabolome of *At. ferrooxidans* it is necessary to develop a protocol that allows us to instantaneously quench the metabolism as well as to extract a large amount of chemically diverse metabolites. The objective of my thesis is to determine the best quenching solution based on its ability to limit metabolite leakage, and the best extraction based on the number of metabolites extracted as well as the recovery values of specific ^{13}C labelled amino acids. Although there are many microbial metabolomics extraction protocols in the literature it is necessary to develop species specific extraction protocols.

Chapter 2: Comparison of Quenching and Extraction Methodologies For Performing Metabolomics in *At. ferrooxidans*

2.1 Introduction

Acidothiobacillus ferrooxidans is an acidophilic chemolithotroph that is found in the acid mine drainage (AMD) environment (Auld *et al*., 2013; Kelly and Wood, 2000; Temple and Comer, 1951). AMD is a extreme environment due to its low pH and high concentration of toxic metals, ferrous iron is an example of one such toxic metal. *At. ferrooxidans* gains energy through oxidization of ferrous iron to ferric iron (Singer and Stumm, 1970), the produced ferric iron is a substrate in an overall abiotic chemical reaction that results in the production of acid (Edward *et al*., 1998; Singer and Stumm, 1970). The production of acid alters the pH of aquatic environments from a near neutral pH to as low as pH 1.0, which kills the plants and animals that are normally present in these environments. In the absence of bacterial metabolic reactions, the production of AMD still occurs but at a much slower rate. Thus the ability of *At. ferrooxidans* to act as a biological catalyst in the production of AMD is, therefore, an environmental concern and is considered the single largest environmental liability in Canadian mining (Mend, 2005). The clear relationship between *At. ferrooxidans*' metabolic processes and environmental degradation makes the metabolome, the suite of small molecule metabolites in this organism (Fiehn, 2001; Oliver *et al*., 1998; Tweeddale *et al*., 1998;) a point of both scientific and industrial interest.

Microbial metabolomics investigations aim to meet four specific criteria: collect sufficient biomass rapidly without altering the metabolome, immediately arrest, quench, the metabolome without damaging the cell, extract all of the metabolites in a cell without

degradation, conversion or metabolite loss, and, finally, analyze the metabolites using an unbiased analytical platform (reviewed in: Xu *et al*., 2014). In practice, performing a microbial metabolomics investigation that meets all four of the aforementioned criteria has been shown to be impossible, the challenge here is to come as close as possible to meeting as many of the criteria as possible. It order to determine how to best meet the four criteria it is necessary to test out different methodologies, keeping in mind the biology of the organism of interest as well as the limitations of the analytical platform being used.

Collecting microbial biomass quickly is generally done through fast filtration, centrifugation, or the use of a rapid sampling device. The cultivation of *At. ferrooxidans*, involves unique challenges due to their biology. Compared to many more commonly studied microbes, this organism grows at relatively low densities as a result of their growth being inhibited by the carbon compounds that they themselves produce as metabolic end products (Baker and Banfield, 2003). These low densities translate into a requirement for the processing of relatively large culture volumes to harvest sufficient biomass for metabolomics analysis. Rapid sampling devices quickly quench metabolism by spraying a small volume of medium into a cold methanol solution (Buziol *et al*., 2002; Schaefer *et al*., 2002; Visser *et al*., 2002). The relatively low density of *At. ferrooxidans* cultures rules out this approach. Similarly, the low density, and high volumes, of *At. ferrooxidans* cultures result in too long processing times, with too poor temperature control, for fast filtration to be effective (Kim *et al*., 2013). Centrifugation, with shorter processing times and greater temperature control, is, therefore, the most appropriate method for collecting sufficient *At. ferrooxidans* biomass for metabolomic analysis.

Immediately following biomass collection, cellular metabolism needs to be halted or "quenched" to prevent biological conversion of the small molecules leading to incorrect characterization of the metabolome. This is generally done through exposure to extreme conditions, most commonly low pH, or extreme temperature as either will inactivate enzymes (Canelas *et al*., 2009; Koek *et al*., 2006; Winder *et al*., 2008). *At. ferrooxidans* is an acidophilic organism so exposure to low pH will not effectively quench its metabolism. We also declined to test high temperature quenching solutions as many metabolites are known to be thermally labile and applying high temperature could result in the degradation of a portion of the metabolome (Gonzalez *et al*., 1997; Marharjan *et al*., 2003; Winder *et al*., 2008). Cold methanol based quenching is the most commonly used form of quenching, however the method is susceptible to cold shock-driven cellular leakage (Wittmann *et al*., 2004). Cold shock results in increased permeability of a cellular membrane as an organism is exposed to rapid decrease in temperature and the increase in cellular permeability is organism specific. Generally, you will see a greater increase in cellular permeability after cold shock in prokaryotes then in eukaryotes and, within prokaryotes, Gram negative bacteria, like *At. ferrooxidans*, will have the greatest increase in cellular permeability (Bolten *et al*., 2007; Wittmann *et al*., 2004). In order to limit metabolite leakage, cold methanol solutions can be cooled to -20°C instead of < -40°C which is the temperature commonly used in *S. cerevisae* applications. The use of -20°C quenching solutions have been shown to reduce metabolite leakage that is especially prevalent in Gram negative bacteria like *At. ferrooxidans*. Furthermore, it has been shown that temperatures between -20°C to -23°C effectively inactivate the enzymes in a variety of mesophilic organisms, and it would be

expected that enzyme resistance to temperature-based inactivation would be the same in all mesophilic organisms (Rabinowitz and Kimball, 2007; Spura *et al*., 2009; Villas Boas *et al*., 2007).

In addition, cold shock associated metabolite leakage can be reduced by buffering the cold methanol solution with an aqueous solution that is adjusted to the same pH and salt concentration that are present on an organism's growth media in order to limit the stress placed on a organisms membrane (Faijes *et al*., 2007; Spura *et al*., 2009; Villas-Bôas & Bruheim, 2007;). A variety of salts have been tested in a variety of microbes and it can be concluded that different salts limit metabolite leakage in an organism-dependent fashion. As a result, it was necessary to test a variety of salts for their ability to limit membrane damage and metabolite leakage caused by cold shock phenomenon in *At. ferrooxidans.*

Extraction solutions also need to be selected with care in order to promote unbiased extraction of a broad suite of chemicals, limit metabolite degradation, and to be compatible with the experimental analytical platform. The analytical platform used in this study is LC-QqTOF, with a HILIC based LC column. Extraction solutions that are compatible with HILIC based LC-MS generally include a mixture of different organic solvents with water (Chauvinet *et al*., 2013; Grata *et al*., 2008). In order to determine which extraction solutions work best for metabolomics *At. ferrooxidans*, it is necessary to test a variety of extraction solutions to determine the number of metabolites that can be extracted, what type of metabolites can be extracted and how well metabolites are recovered.

Microbial metabolomics protocols are surprisingly species-specific and validated protocols are fundamental for comparison of metabolomics results across research groups and species. Most validation research has, however, focused on neutrophilic model organisms (Mushtaq *et al*., 2013) with comparatively little attention to microbial extremophiles. The objective of this study is to develop and validate a functional protocol that can be used to perform metabolomics on *At. ferrooxidans*, an acidophilic chemolithotroph. We present here a quenching protocol that effectively halts the metabolism of *At. ferrooxidans* with minimal leakage, as well as an extraction protocol that extracts a large number of metabolites and recovers metabolites as successfully as possible.

2.2 Methods

2.2.1 Strain, Media and Cell Culture Conditions

Acidithiobacillus ferrooxidans (American Type Culture Collection 23207) was grown in 1 L of Tuovinen and Kelly liquid medium ([$FeSO_4 \cdot 7H_2O$] = 33.4g/L; 0. 3% (w/v) $(NH_4)2SO_4$; $MgSO_4 \cdot 7H_2O$; K_2HPO_4, pH 2.5) (Tuovinen and Kelly, 1973). Cells were incubated on a gyratory shaker at 180 rpm and 30 °C.

2.2.2 Growth Rates

The growth rates of *At. ferrooxidans* were determined in order to properly understand the organism's growth dynamics and ensure that *At. ferrooxidans* cells used in this study were always in the late exponential phase of their growth which corresponds to an optic density (OD) of 0.85 for the protocol validation measured at 425nm. The biological comparison consisted of comparing the bacterial cells in their late exponential growth phase (OD_{425} = 0.85) to the bacteria in their early exponential growth phase

(OD_{425} = 0.65). The growth curve was constructed using the natural logarithm of the concentration of ferric iron produced as a proxy for the bacteria's growth as described by Karamanev *et al*., (2002) (Figure A1).

2.2.3 Sample Collection

Two liters of cells were grown to an OD_{425} of 0.85, mixed together (it was not possible to culture 2L of media in our incubator) and centrifuged at 14000 g for 5 minutes at 4 °C. The pellet was then transferred into a 1.5 mL microcentrifugation tube and centrifuged for an additional minute at 15682 g at 4 °C. A 40 µL aliquot of cell supernatant was removed for footprint analysis and then one of three quenching solutions were added.

2.2.4 Footprint Analysis

The aliquot of un-quenched supernatant (above) was filtered through a syringe filter (0.22 µm; Millipore express) to remove any cells that didn't pellet during centrifugation and ensure that there were no cells present in the footprint sample. The filtrate was then immediately snap frozen on dry ice and stored at -80 °C for later analysis. In this analysis, the aliquot was evaporated to dryness and then stored at -80°C until it was ready for analysis and then it was reconstituted to the same volume (40 µL) in 80:20 (acetonitrile:water) in order to ensure the sample was compatible with a HILIC analysis (Figure 1).

2.2.5 Quenching of Microbial Biomass

The efficacy of three different quenching solutions was quantified using a common quenching protocol. 40 µL of one of the quenching solutions was added to 40 µL of cell culture and the mixture vortexed to ensure complete mixing. The quenched

cells were centrifuged for 5 minutes at 2319 g and 4°C and the supernatant removed, filtered through a syringe filter (0.22 μm; Millipore express) to remove cell debris, and then snap frozen in liquid nitrogen until further analysis. Prior to analysis, the aliquot was evaporated to dryness and then reconstituted to 40 μL in 80:20 (acetonitrile:water) in order to ensure the sample was compatible with a HILIC analysis.

After removal of the cell media and quenching solution, the cells were divided into two samples and placed on dry ice for the rest of the extraction process. 2 μL of ^{13}C labelled amino acids were added to one sample of the cells, the "after quenching" (AQ) sample. Two μL of ^{13}C labelled amino acids were added to the other cell sample, known as the "before analysis" (BA) sample immediately prior to analysis. Comparison of the metabolites in these two cell extracts allows us to assess degradation of metabolites during the extraction procedure (Figure 1).

2.2.6 Quenching Solutions

Three different quenching solutions were tested. All quenching solutions consisted of a 60% MeOH and 40% H2O (v/v) with 0.85% (w/v) of one of the buffering agents, adjusted to pH 2.5. The three buffering agents were ammonium formate, physiological saline and a modified version of the salts used in TK media (which consisted of equal amounts of $MgSO_4$ and NH_4SO_4).

2.2.7 Extraction Solution

Three extraction solutions were tested, Chloroform:Methanol:H_2O (1:3:2), acidic acetonitrile water (4:1) pH adjusted with 10mmol formic acid and, Isopropanol:Methanol:H_2O (3:3:2). The extraction solutions were added immediately after quenching and the solutions were at – 20°C when added, after adding the solutions

the cells were vigorously vortexed and then centrifuged at 15682 g for ten minutes. The extraction liquid was then collected and placed at -80°C, until further analysis (Figure 1).

2.2.8 Experimental design

Three methods of quenching microbial metabolism were employed and compared in combination with three methods of extracting metabolites for a total of nine potential microbial metabolomics methods. Seven replicates of each potential method were performed giving us results from sixty-three biological samples (Figure 2). Each of the sixty-three samples consisted of four different aliquots: the footprint/cell supernatant analysis, the cell supernatant + quenching solution and the BA and AQ sample (Figure 1).

2.2.9 Randomization of Microbial Growth

The cultures required for one set of biological replicates for nine different microbial metabolomics protocols were grown over the course of three days. Within each set of biological replicates randomization was re-done, this randomization ensured that there was no bias based on when a culture was grown (Figure 2).

2.2.10 Randomization of Analytical Runs

Numerous analytical runs needed to be done in order to carry out this experiment. An analytical run is defined as the time from when we started running the first sample on the LC-MS until the time we finished running the last sample on any given day. In order to limit degradation, samples were only loaded on the LC/MS for ten hours, and a single sample takes fifty-five minutes to run on the LC-MS. The first section of analytical runs consisted of the first biological replicate of the nine potential microbial metabolomics protocols, in total there were thirty-six samples to be run. In order to keep analytical runs under ten hours all thirty-six samples were run over the course of three days. In all seven

sets of biological replicates samples were randomized to determine which day they would be run on, and which order they would be run in during the ten hour LC/MS run (Figure 2).

2.2.11 Technical Replication

In order to ensure our LC-MS method was robust, it was necessary to assess the repeatability of the experiment within a ten hour LC/MS run. In order to assess within-run repeatability for each measurement taken, there were three samples that were randomly selected to be run in triplicate on randomly selected days. In total, three cell supernatants were run in triplicate on three different days, nine cell supernatants with quenching solution (three of each individual quenching solution) were run in triplicate on different days and finally, nine of each extraction solution. The within-run replicates were used to see if the number of metabolites present varied substantially based on when a sample was run on the LC-MS on any given day. In addition, the ^{13}C internal standards were quantified to see if our quantification was biased based on when samples were run. Our results show that there was very little variation in our analytical runs in terms of measurement of the number of metabolites as well as quantification of the ^{13}C labelled internal standards (Figures A2-A10).

2.2.12 Reagents

All metabolite standards were obtained in high purity from Sigma Aldrich (St. Louis, MO) or Bioshop (Burlington, ON). All solvents were high purity LC-MS grade and were obtained from Fisher Scientific (Sunnyvale, CA).

2.2.13 Instrumentation

All experiments were carried out on a Dionex Ultimate 3000 Rapid Separation LC system (Thermo Scientific, Sunnyvale CA) coupled to a Bruker micrOTOF-Q IITM Electrospray ionization-quadrupole-time of flight mass spectrometer (Bruker Daltonics, Billerica MA). The MS was operated in positive mode and was calibrated using sodium formate dissolved in water/isopropyl alcohol, infused at a flow rate 0.18 mL/hour using a KD scientific 5 mL infusion pump. The ionization source working parameters were as follows: capillary voltage 4 kV, ion energy of quadrupole 4 eV/z, dry temperature 200 °C, nebulizer 4.0 bar, and dry gas 9.0 L/minute. Chromatographic separation was achieved at 0.400 mL/minute using a SeQuant ZIC- pHILIC 150 x 2.1 mm 5 μm polymeric attached to a SeQuant ZIC-pHILIC 20 x 2.1 mm guard column.

2.2.14 Liquid Chromatography Gradient

The LC running buffers used were acetonitrile with 0.1% formic acid (v/v) and water with 20 mmol Ammonium formate. The LC mobile phases used are: A-20 mmol ammonium formate solution in LC-MS grade water, B 100% ACN with 0.1% formic acid. HPLC gradient used: (t=0-1 minute) 3% A, 97% B;(t=24 minute) 40%A, 60%B;(t=28 minute) 40%A, 60% B (t=33 minnutes) 3% A, 97%B; (t=34minutes) 3%A, 97% B. HPLC flow rate 0.4mL/minute.

2.2.13 Data Analysis

Data were acquired using the Hystar 3.2 software package and evaluated using Compass Data Analysis 4.0 software package (Bruker Daltonics, Billerica MA). Principal component analysis (PCA) and t-tests were performed using ProfileAnalysis 2.0 software (Bruker Daltonics, Billerica MA). Metabolite peaks were detected and time

aligned using the Find Molecular Features function (FMF) in DataAnalysis software works in conjunction with the ProfileAnalysis software. The analysis window used in these three analyses commenced at 0.2 minutes and stopped at 35 minutes. Features were represented as buckets, consisting of a mass to charge ratio (m/z) and a retention time. Each bucket was normalized by the sum of all buckets in the analysis, Pareto scaling was used. Bucket intensity values of the top 30 features, ranked by T-test/ANOVA, were exported into Metaboanalyst software (Xia *et al*., 2009, 2012), which was used for hierarchical clustering using a Spearman rank correlation and an average clustering algorithm.

2.3 Results and Discussion

2.3.1 Choice of Quenching Methods Tested

We tested three quenching solutions, each a variation of cold methanol based quenching (de koning and van Dam, 1992) with each varying in the type of salts present, either ammonium formate, physiological saline, or a modified version of TK media. Ammonium formate was selected to match the aqueous phase of our liquid chromatography running buffers and using it in our quenching procedure could limit the formation of unwanted metal adducts associated with using more than one salt (Faijes *et al*., 2007). Physiological saline was selected because it is a salt that has been used as part of quenching solutions in a large variety of microorganisms, the fact that it decreases membrane damage in a numerous microorganism (Spura *et al*., 2009; Wang *et al*., 2014) suggested it could potentially work well with *At. ferrooxidans*. Lastly, modified TK salts media was selected because it is the growth media we use for *At. ferrooxidans* and using

the same media for both growth and quenching has been found to limit cold shock in other systems (Hoque *et al*., 2005; Oldiges *et al*., 2004).

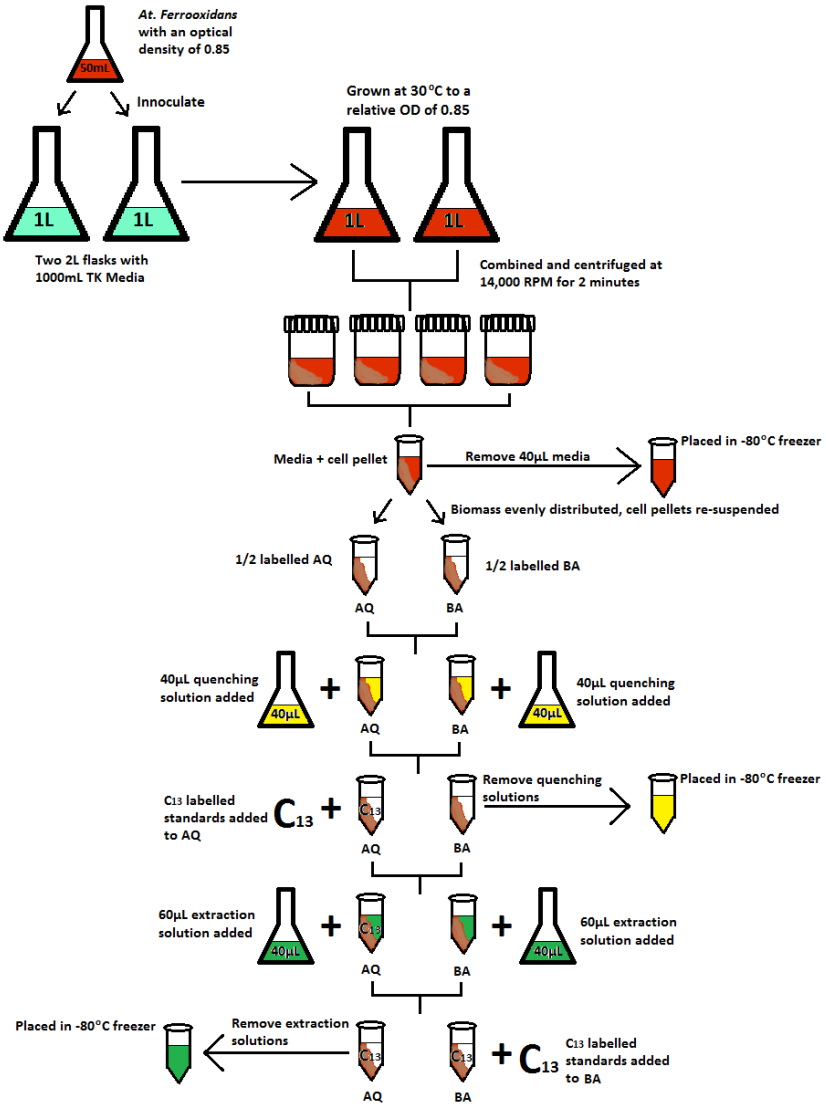

Figure 1: **Schematic of the quenching and extraction procedures**.

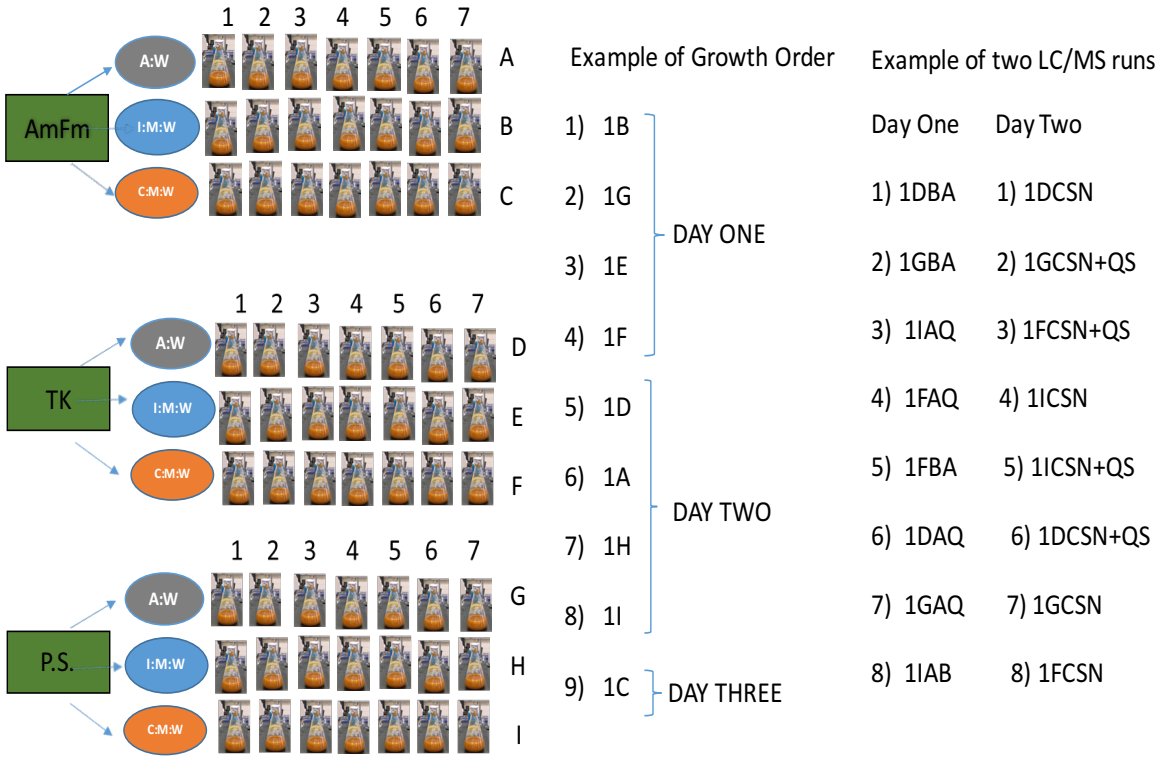

Figure 2: **Schematic description of biological replication and technical randomization**. Each biological replicate e.g. 1A or 5G goes through the protocol shown in Figure 1.

2.3.4 Leakage of Metabolites

Of the three quenching solutions tested, the ammonium formate solution performed the best with the most limited amount of metabolite leakage. With the ammonium formate based quenching solution only seventy-one metabolites leaked into the cell supernatant. To contrast that with the other quenching solutions, both modified TK salts and physiological saline resulted in double that amount of leakage (Figure 3).

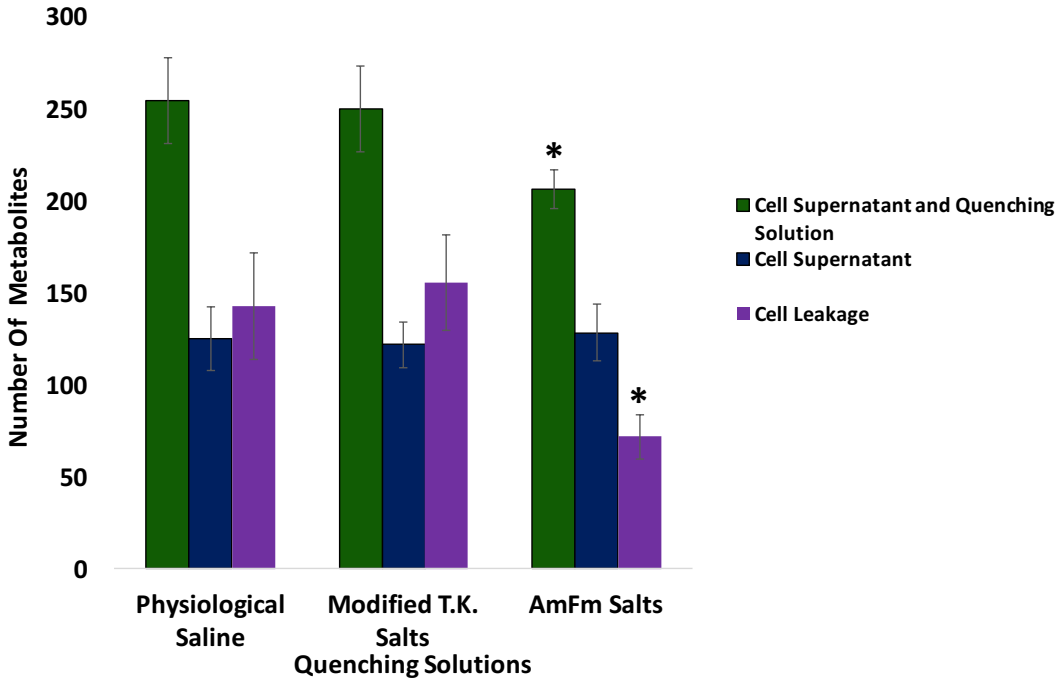

Figure 3: **Mean cell leakage across treatments.** The asterisks denote that there is significantly less metabolite leakage when using the AmFm salts.

2.3.5 Choice of Extraction Method

The three chosen quenching solutions were tested in combination with three extraction solutions: acidic acetonitrile water (AAW), isopropanol:methanol:water (IMW), and chloroform:methanol:water (CMW) (Figure 2).

We tested AAW and IMW as extraction solutions because they have both been shown to effectively extract a broad suite of metabolites in other Gram negative organisms (*E.coli* and *S.degradans*) (Rabinowitz and Kimball *et al*., 2007; Shin *et al*., 2010) and are compatible with the analytical platform being used and as such could work well as extraction solutions in *At. ferrooxidans*. AAW is especially noteworthy because it

is effective in limiting the degradation of nucleotide triphosphates (Rabinowitz and Kimball *et al*., 2007). The degradation of nucleotide triphosphates skews interpretation of the endometabolome, and causes the misestimation of an organism's cellular energy charge ratio (Rabinowitz and Kimball *et al*., 2007). Interestingly when the AAW extraction was used in *S. cerevisae* it was not able to more effectively recover nucleotide triphosphates and overall produced worse results as compared to the chloroform:methanol extraction (CM) (Canelas *et al.,* 2009). The discrepancy in AAWs ability to outperform other extraction methods is species specific, and the ability of CM to outperform AAW led to us testing a CM based extraction solution. CM based extraction solutions have been criticized due to their inability to effectively recover polar compounds (Winder 2008; Zaldivar *et al*., 2002). *Drosophila melanogaster* metabolomics studies have seemingly remedied CM's inability to extract polar compounds by using a CMW extraction solution that consists of a very small amount of chloroform and includes water resulting in 1:3:2(Chloroform: Methanol: Water) extraction solution that extracts polar compounds (Knee *et al*., 2013).

Across all three quenching methods, AAW and IMW resulted in the extraction of a larger number of compounds than CMW (Figure 4-6). Also across all three quenching methods, IMW resulted in an average extraction of more metabolites than the AAW method, although this trend was not statistically significant. In order to determine if this trend would be statistically significant if a larger sample size had been used, we pooled all the extraction solutions together regardless of quenching method used, effectively tripling the sample size. Across these pooled extractions, the IMW extraction method did, in fact, result in the greatest number of peaks being extracted and detected (Figure 7). The

IMW method resulted in an average of 105 more peaks being detected than the AAW extraction method (Figure 7). On average the IMW extraction method yielded 870 metabolites, twelve percent more than the AAW method. The AAW method yielded 765 metabolites, thirteen percent more than the CMW method. Finally, the CMW method yielded 664 metabolites, twenty-four percent less than the IMW method (Figure 7). In addition, we were able to specifically identify a number of metabolites based on comparison with one hundred metabolite standards. Using the IMW extraction protocol we identified fourteen of these metabolites, nine using the AAW protocol, and six using the CMW protocol (Table 1)

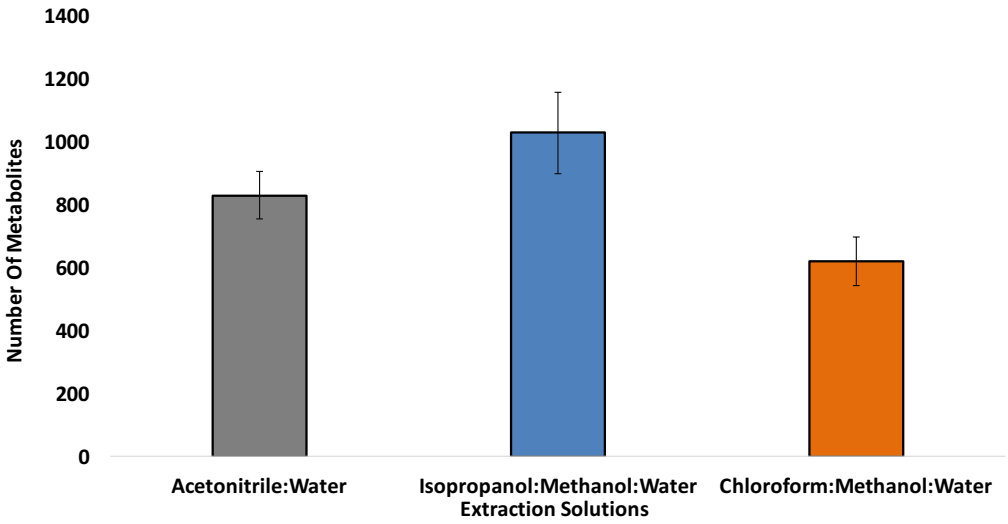

Figure 4: Mean number of metabolites extracted using three different extraction solutions after quenching with ammonium formate.

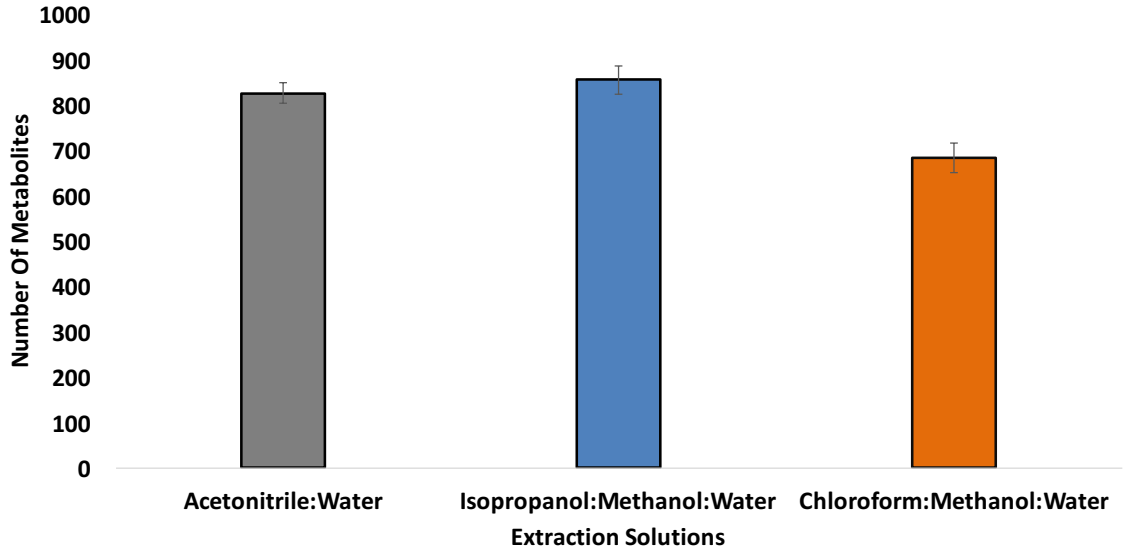

Figure 5: Mean number of metabolites extracted using three different extraction solutions after quenching with physiological saline.

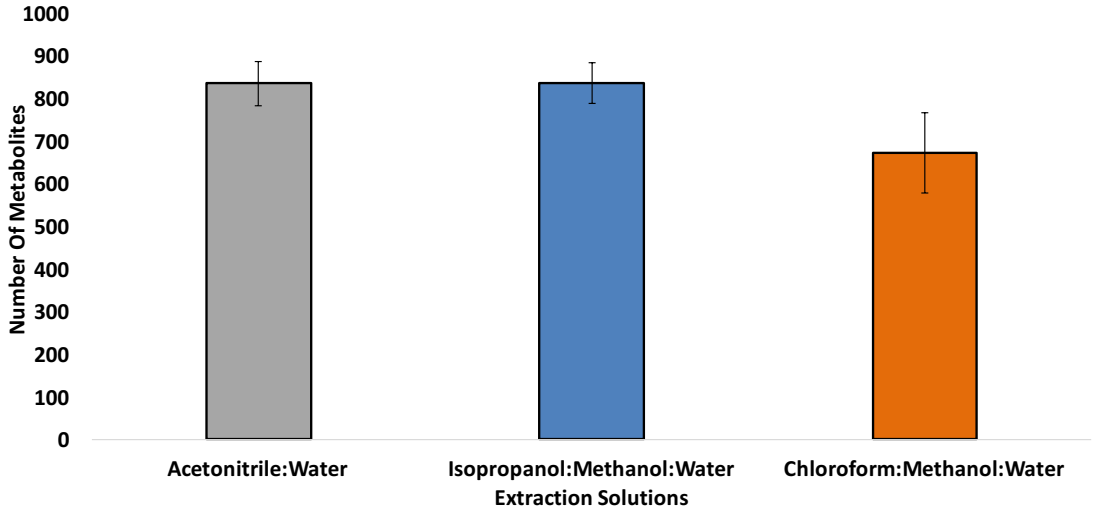

Figure 6: Mean number of metabolites extracted using three different extraction solutions after quenching with Tk salts.

Table 1: List of endogenous metabolites present or absent in bacteria based on extraction method.

Metabolite	IMW	AAW	CMW
Putrescine	Present	Absent	Absent
Valine	Present	Present	Absent
Nicotinamide	Present	Present	Present
Acetyl Co-A	Present	Absent	Absent
Isoleucine	Present	Present	Present
B-glycerophosphate	Present	Absent	Absent
Threonine	Present	Present	Absent
Lactobionate	Present	Absent	Absent
Spermidine	Present	Absent	Present
Betaine	Present	Present	Absent
Heptanoic Acid	Present	Present	Absent
Cystine	Present	Present	Absent
Cytidine-5'-diphosphocholine	Present	Present	Present
Proline	Present	Present	Present
Uric Acid	Absent	Absent	Present

Overall, we detected and identified the largest number of metabolites using the IMW solution, consistent with the research done on *S. degradans* (Shin *et al.,* 2010) and with widespread use of methanol based extraction solutions (e.g. Shin *et al*., 2010; reviewed in Xu *et al*., 2014). It is important to note that although we included AAW in this study because of its ability to extract triphosphorylated intermediates (ATP, ADP, AMP etc…) in other systems (Rabinowitz and Kimball, 2007), we were not able to detect triphosphorylated intermediates in *At. ferrooxidans* using AAW. It is possible, however, that triphosphorylated intermediates in *At. ferrooxidans* do extract well in AAW they were just below the limit of detection of our analytical instrument due to the extremely low biomass used in this study. Finally, the lowest number of peaks were seen when CMW extraction was employed (Figure 7). This result is consistent with numerous

studies done on different microbes that use chloroform in the extraction solvent (Faijes *et al*., 2007; Zaldivar *et al*., 2002). Chloroform-based methods are often avoided in microbial metabolomics research due to the toxicity of the solvent and due to the fact that it isn't compatible with certain ion sources. Our results show that the use of a method containing chloroform is not ideally suited towards a global *At. ferrooxidans* extraction. Overall chloroform-containing methods should be avoided for *At. ferrooxidans* metabolomics studies unless it is necessary to detect lipids.

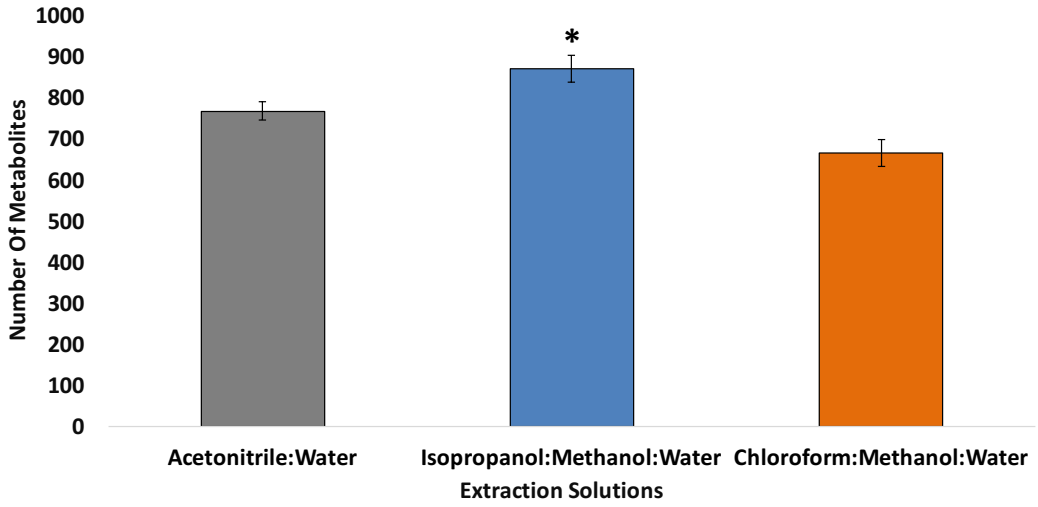

Figure 7: Mean number of metabolites extracted using three different extraction solutions after the results of all three quenching solutions were pooled together. Asterisk denotes that there are significantly more metabolites in the isopropanol:methanol:water extraction solutions.

2.3.6 Recovery of ^{13}C Amino Acids

We also specifically quantified the effectiveness of each extraction solution for a variety of amino acids using ^{13}C labelled standards. Amino acids were added immediately after quenching and immediately prior to analysis. Adding ^{13}C labelled standards immediately prior to an analytical analysis allowed us to quantify the ^{13}C standards while

including matrix effects, while adding ^{13}C labelled standards immediately after quenching allows us to assess how much a compound is degraded or lost through an extraction method while still taking into account matrix effects (Birkmeyer *et al*., 2005; Canelas *et al*., 2009) (Figure 1). ^{13}C standards were chosen to be added to a cell because they behave the same way as endogenous compounds but we can differentiate added and endogenous compounds using mass spectrometry (because MS can easily differentiate ^{12}C from ^{13}C). In model organisms (e.g. *E.coli* and *S.cerevisae*), endogenous compounds can be labeled in vivo, by growing the microorganisms in media containing ^{13}C labelled substrates (Birkmeyer *et al*., 2005; Canelas *et al*., 2009). Such in vivo labelling allows for the assessment of the recovery of all metabolites within an organism rather than a select few. However, *At. ferrooxidans* does not take up organic carbon and has extremely heterogeneous growth making such in vivo labelling using ^{13}C stable isotopes of multiple biological replicates impractical (Liu *et al*., 2011) requiring us to use in vitro labeled metabolites. We selected ^{13}C labelled amino acids because they resolve well on a HILIC column, represent a variety of diverse chemicals, are important parts of metabolic processes (Bolten and Wittman, 2008). Figures 8-10 show the amount of ^{13}C labelled amino acids that are present in IMW, AAW and CMW immediately after quenching and immediately prior to analysis.

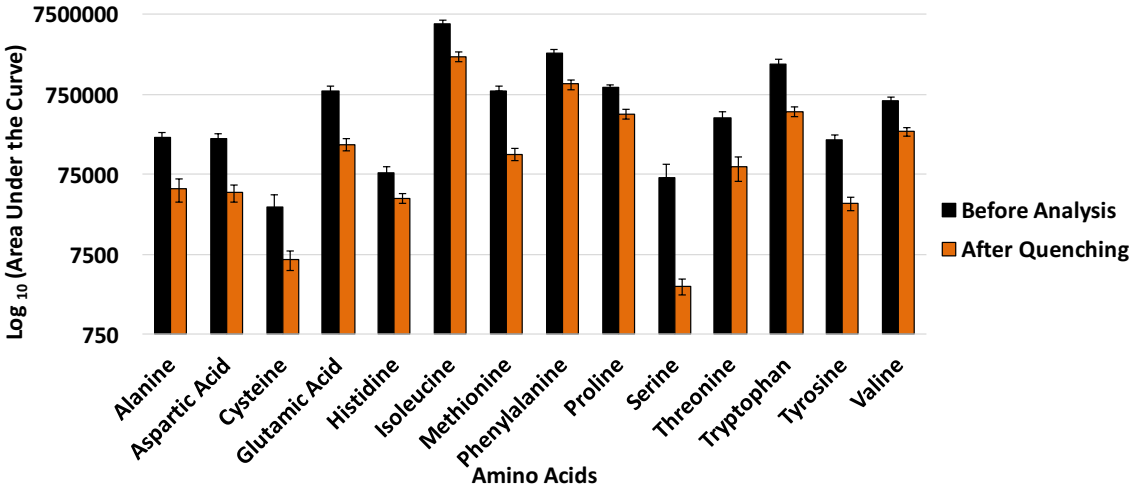

Figure 8: The log transformed mean of the area under the curve of fourteen amino acids that undergo the chloroform:methanol:water extraction procedure.

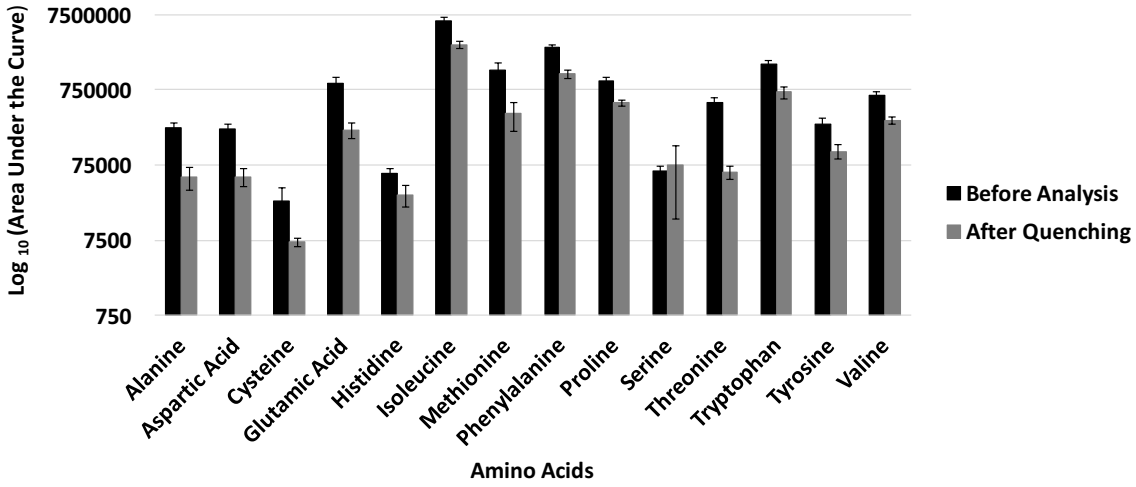

Figure 9:The log transformed mean of the area under the curve of fourteen amino acids that undergo the acetonitrile:water extraction procedure.

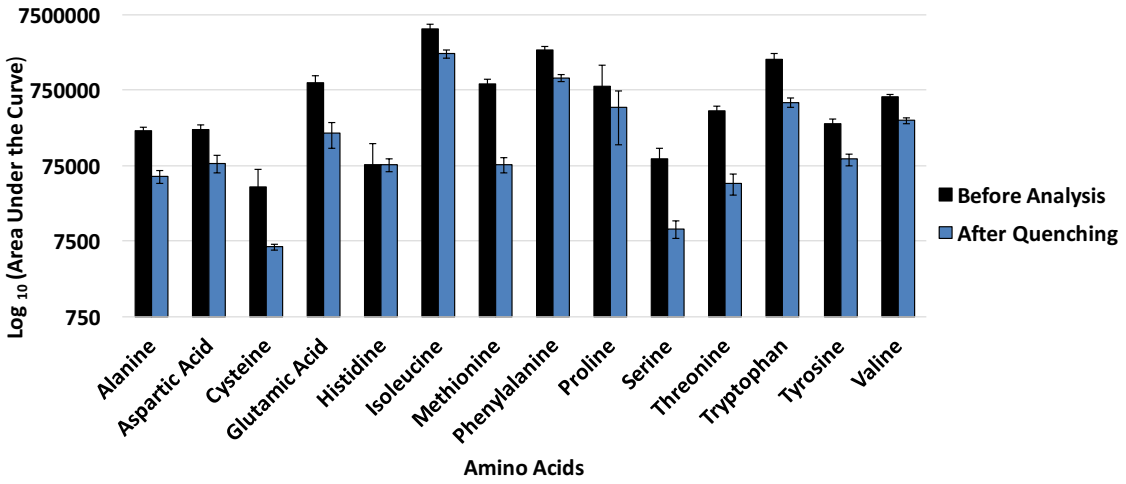

Figure 10: The log transformed mean of the area under the curve of fourteen amino acids that undergo the isopropanol:methanol:water extraction procedure.

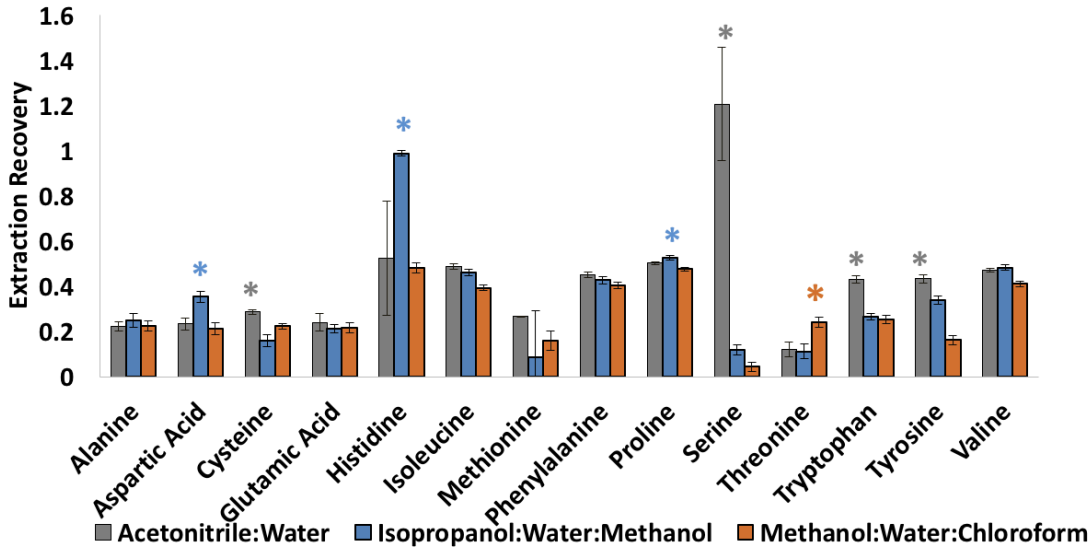

Figure 11: Mean extraction recovery of fourteen ^{13}C labelled amino acids as determined by the equation BA/AQ. The asterisks represents a recovery that is significantly higher in one extraction solution, the colour of the asterisks correlates to the extraction solutions in the legend.

Overall, IMW and AAW did the best job of effectively recovering all ^{13}C labelled metabolites. CMW consistently resulted in a lower recovery of many amino acids except in the case of threonine which was recovered at a much higher rate than either of the other methods tested (Figure 11). In addition, although both IMW and AAW worked well overall they effectively recovered very different classes of amino acids. AAW did

the best job recovering serine, tryptophan, tyrosine and cysteine (Figure 11). All four of these amino acids contain polar neutral and slightly polar neutral side chains (Nelson *et al*., 2008). IMW was able to recover aspartic acid, proline and histidine in a higher amount than in the AAW solution (Figure 11). IMW is able to extract a wider variety of amino acids including basic polar, acidic polar and neutral non-polar amino acids (Nelson *et al.,* 2008). These results corroborate the results of many other researchers which show that it is impossible to extract all metabolites completely with one extraction solution. Our results also show that IMW is a better extraction solution for a broad based analysis because it is able to recover a variety of metabolites with of different chemical natures. It is important to note that AAW would be a better extraction solution to use if a researcher was interested in more polar/slightly polar metabolites.

2.4 Conclusion

To conclude the ammonium formate quenching solution is the best solution to stop the metabolism of *At. ferrooxidans* because it causes the least amount of metabolite leakage. Furthermore, the best extraction solution IMW because it allows to extract the most metabolites, is reproducible and allows to effectively recover the most diverse group of amino acids.

Chapter 3 Future Directions

3.1 General Conclusions and Future Work

We have developed a general quenching and extraction methodology that can be used to perform metabolomics studies in *At. ferrooxidans*. Future work using this protocol will follow two directions: biological and further improving method performance. Now that we have a method that works, we can use this method to ask questions about *At. ferrooxidans* metabolism and that of the broader AMD microbial community. However, although we have developed a method that works well in *At. ferrooxidans*, there is still room to improve. Method development is always an ongoing task in metabolomics in part due to the chemical complexity involved, as well as due to the constant increase and innovation in the analytical technology as well as the availability of more broad based columns.

3.2 Expanding Metabolite Identification

Currently, we can analyze 1,000 mass signals in *At. ferrooxidans,* however we can only name about 14 metabolites. In order to truly understand the metabolome of *At. ferrooxidans* we need to increase the number of metabolites we can name. One of the ways to increase the number of metabolites we can name is through the use of tandem mass spectrometry; the identification of metabolites through fragmentation of parent ions. A chemical fragmented at a certain energy will always break apart in a specific way leaving a characteristic fragmentation pattern for that metabolite. There are numerous online databases that contain the fragmentation patters for thousands of metabolites, and that number is constantly increasing (Tiller *et al*., 2008). Now that we have a reliable extraction and analysis protocol in place we can use fragmentation patterns, accurate

mass and retention time in order to confidently name metabolites. Currently we name metabolites by comparing accurate mass and retention time to that of a pure standard solution, using a pure standard solution. Using a pure standard solutions can be incredibly expensive. Using fragmentation patterns is a much more cost effective way of naming metabolites, however it does have the drawback of being incredibly time consuming, and it will only work with one method. A single method will consist of using a specific column, with two specific mobile phases that will run through the column in various amounts as dictated by a pre-determined gradient. If you identify the fragments of a metabolite with a specific mass eluting at a specific time using any one method, anytime you change the method, you need to redo the tandem MS in order to make sure you are still identifying the same metabolite. We are currently using one method in all of our metabolomics analysis. In the future we will continue to use that same method for our metabolomics analyses as well as perform tandem MS, to create a Merritt lab database of fragmentation patterns that we will compare to fragmentation data that is available on the internet. In doing so we will be able to slowly increase the number of metabolites we can name in a analysis.

3.3 Expanding Coverage of the Metabolome

In the current study, we looked at how extraction protocols affected the recovery of polar metabolites. In the future, we can expand our coverage of the metabolome by using columns that resolve less polar as well as non-polar metabolites, or that can differentiate between molecular isomers. Non-polar and less polar metabolites can be resolved on a C_{18} based column and isomers can be differentiated on porous graphitized carbon columns (Wan *et al*., 1996). There is currently an abundance of literature

revolving around the detection and resolution of non-polar and less polar metabolites. In addition it is known that left handed amino acids and right handed sugars make up the majority of what is present in the metabolome so it is unclear how much information can actually be gained from being able to resolve isomers (Hein and Blackmond, 2012; Yanes *et al*., 2011). For the two aforementioned reasons it would be easier and we would probably gain more knowledge from developing a C_{18} based column method first. In order to develop a method with the use of C_{18} based column, we will need to purchase a variety of non-polar standards that are found ubiquitously in prokaryotic life forms. The ability to run and confidently identify non-polar standards will allow us to develop a method (pick a column and gradient) that effectively separates non-polar metabolites.

3.4 Increasing Sensitivity to Identify Metabolites Present at Low Concentration

In the Merritt Lab we currently use a MICROTOF-QII which is an extremely sensitive instrument used in order to identify metabolites, however more sensitive technologies do exist. The term sensitivity in relation to hyphenated mass spectrometry techniques refers to the smallest amount of a chemical that we can detect with some degree of confidence and can also be referred to as the detection limit (Pardue *et al*., 1997). Working with a LC-MS that is as sensitive as possible is extremely important in *At. ferrooxidans* metabolomics because *At. ferrooxidans* is a microbe that is difficult to cultivate in a lab and so we are only able to obtain a small biomass, which results in the extraction of many metabolites that are present at a low concentration. Metabolites that are present at a very low concentration may be below the detection limit of the LC-MS, making it impossible to differentiate between whether a metabolite is absent in *At.*

ferrooxidans or there just isn't enough of it to detect. Working with a more sensitive instrument would allow us detect more metabolites and therefore have a better understanding of the metabolome of *At. ferrooxidans.*

A Waters G2-XS Qq-TOF is a more expensive instrument that is available and it has 50-100 times increased sensitivity (Waters, 2012). The increased sensitivity is due to changes in ion optics. Ion optics refers to the process of getting charged particles from the ion source to the mass analyzer. In order to get the best sensitivity possible, it is necessary to optimize the transfer of ions to the mass analyzer so as many charged particles get to the ion source as possible (Orloff, J., 2008). The MicroTOF QII uses a dual ion funnel to move as many ions as possible from the ion source to the mass analyzer. The improvement in Waters G2-XS Qq-TOF is due to a change in ion optics. The Waters Qq-TOF utilizes new technology that not only directs the charged particles into the instruments but also extracts the neutral particles (which mostly originate from the excess solvent) to the exhaust, the neutral particles contribute to background noise in chromatograms. The decrease in background noise improves the signal to noise ratio of metabolite which means we can detect a smaller amount of a chemical and therefore the instrument is more sensitive (Waters, 2012). As analytical technology progresses and becomes more affordable we will be able to use more sophisticated technology in our own lab and using more sophisticated technology will allow us to work better with samples that contain a low biomass. With an increased sensitivity we should be able to identify more molecular features as well as name more metabolites.

Not only would a more sensitive LC-MS allow us to detect more metabolites it could also make working with *At. ferrooxidans* easier. We currently have to cultivate

two liters of media in order to have enough biomass to detect metabolites on the MicroTOF QII, working with a more sensitive machine would allow us to use less media which would mean we could grow bacteria more easily and also potentially develop a rapid sampling technique.

3.5 Rapid Sampling Technique

In terms of method development, one area that is difficult to deal with in *At. ferrooxidans* is developing a rapid sampling technique. A rapid sampling technique is frequently defined as being able to collect an organism and stop its metabolism in 1-2 seconds (Villa Boas, 2007). Currently researchers have engineered their own automated sampling systems in order to collect cells quickly and stop their metabolism. These sampling technique have been applied successfully to a wide variety of sample types including mammalian cells, *S. cerevisae* and *E. coli* (Buzziol *et al*., 2002; Schaefer *et al*., 2002, Visser *et al*. 2002). All rapid sampling devices that have been developed to date are relatively similar. The basic design consists of tubing that is connected to a bioreactor or fermentor, the cells are pumped through that tubing in an automated fashion. Along the tubing there is a series of sampling ports that allow a controlled amount of sample to be sprayed into a pre-cooled quenching solution. The first sampling port is located immediately outside the bioreactor and allows for sampling to occur at time zero. After the first sampling port a second tube allows a perturbing agent to enter the system, where it mixes with the cells. The cells mixed with the perturbing agent then are pumped through the tubing at specific flow rate, and at each sampling port they are sprayed into a quenching solution. The sampling ports are located at sequential locations allowing the flow rate to determine how often things are sampled. A automated fast sampling system

can allow a researcher to do experiments where you check the changes in a cells in reaction to a perturbing agent every *n* number of seconds, allowing a researcher to watch metabolic changes over time, while ensuring that metabolism is quenched very quickly so that the metabolites being extracted are representative of the metabolome of cell under a certain perturbation (Buzziol *et al*., 2002; Schaefer *et al*., 200; Visser *et al*., 2001). Applying a rapid sampling technique such as coupling an online sampling device to a bioreactor would make future experiments in *At. ferrooxidans* much easier to conduct, and allow us to quench the metabolism much faster resulting in a metabolome that is more representative of *At. ferrooxidans*. In addition, working with an automated system would eliminate human error thereby minimizing the variance in biological replicates of the same sample type. The main issue that we face when working with *At. ferrooxidans* is that we need to work with a very large amount of media (2L) in order to obtain enough biomass for an analysis. It is impossible to spray 2L of media into a quenching solution in an automated fashion, therefore it is necessary to decrease the amount of media being used. In order to decrease the amount of biomass we need for one sample we can either work with a more sensitive instrument or grow *At. ferrooxidans* with *Aph. Acidophilium* in co-culture which will result in a higher biomass due to syntrophic interactions (de koning and van Dam, 1992; Liu *et al*., 2011; Wu *et al*., 2010).

3.6 Understanding Community Dynamics

At. ferrooxidans is one member of the dynamic AMD microbial community (Auld *et al*., 2013; Strenten-Joyce *et al*., 2013; Tan *et al*., 2009). The AMD environment is relatively extreme, resulting in a simpler microbial community as defined by species richness, than in a more neutrophilic environment (Biers *et al*., 2009; Mohit *et al*., 2015).

The simpler AMD microbial community contains hundreds of different species instead of thousands or tens of thousands of different species that would be present in a more neutrophilic environment (Auld *et al*., 2013; Biers *et al*., 2009; reviewed in Méndez-Garcıa *et al*., 2015). The presence of simpler microbial community makes the AMD environment a good starting point for understanding microbial community dynamics for two reasons; a community with less members will be easier to reconstruct in a lab, and having less community members makes it easier to tease out the role of each member. Metabolomics is a good research tool to use for understanding community dynamics because it is sensitive to both upstream (genomic, proteomic and transcriptomic) signals and downstream (abiotic and biotic environmental) signals. A community metabolomics footprint will be in part a reflection of the upstream processes in an organism/community and will therefore reflect the genetics of an organism/community but it is also very flexible, and will react to the downstream environmental processes, such as the addition of new organisms (Schmidt *et al*., 2004).

In order to ask questions about bacterial community dynamics one must first reconstruct a bacterial community. The most manageable bacterial community to reconstruct is a bacterial community with just two members. A future experiment could start off by determining the individual metabolomic fingerprint of two species and then growing them together to determine their combined metabolic fingerprint. When working with the AMD community the two organisms that should be worked with first are *At. ferrooxidans* and *Acidophilium acidophilum*, due to the fact that they have a mutually beneficially relationship that will result in more biomass allowing us to alleviate some of the sensitivity issues that have been mentioned in section 3.4 and 3.5. In

addition, there is currently a plethora of information in the literature about the synergistic interactions between *At. ferrooxidans* and *Aph. acidophilum* as well as the necessary growth conditions for these organism, which will allow us to easily grow these bacteria and correlate out metabolomics results with other data (Baker and Banfield, 2003; Harrison *et al*., 1984; Liu *et al*., 2011). To briefly summarize the metabolic interactions of *At. ferrooxidans* and *Aph*. *acidophilum*; organic carbon is known to inhibit the growth of *At. ferrooxidans*. *Aph*. *acidophilum* consumes the organic carbon that is present alleviating or reducing its inhibition of *At. ferrooxidans*. Similarly, *At. ferrooxidans* alleviates thiosulfate inhibition of *Acidophilum* by consuming it through oxidization reactions (Gurung and Chakraborty 2009; Harrison, 1984; Marchand and Silverstein 2003).

In order to build a synthetic community of bacteria that resembles the AMD environment it would be logical to order bacteria from the American type culture collection (ATCC), as those bacteria are easy to obtain, and grow well in laboratory conditions. It also may be prudent to specifically use *Aph. Acidophilum* ATCC 27807 and *At. ferrooxidans* ATCC 23270 because there has been some work done on the growth dynamics of these organism. The term growth dynamics refers not only to these organisms' growth curves, but also to the number of cells belonging to each species and their ratio to each other. In the literature the initial inoculation ratio of a co-culture was 72:1 *At. ferrooxidans* to *Aph. acidophilum*. The initial ratio changed substantially throughout the 264 growth period with its extremes ranging from 42:1- 201:1 *At. ferrooxidans* to *Aph. acidophilum* (Liu *et al*., 2011). The most interesting experiment may be to take multiple samples throughout this communities' growth curve, and attempt

to see how the community changes metabolomically over time. The metabolomic data obtained can be related to the ratio of the cells that are present, and each organism's individual growth curve. Previous research on this system shows that the exponential phase of *Aph. Acidophiliums* growth lags behind *At. ferrooxidans* exponential growth phase. The time difference between the start of each organisms' exponential growth phase has been correlated with gene expression data from genes known to be involved in energy acquisition, and shows that in a carbon absent environment *Aph. acidophlium* relies on being able to consume the carbon produced by *At. ferrooxidans* metabolism (Liu *et al*., 2011).

At the moment we currently have some understanding of the metabolic interactions of these two organisms, e.g. the reciprocal reduction in carbon and thiosulfate inhibition (Harrison 1984; Marchand and Silverstein 2003), and this data has been correlated to transcriptional changes for genes known to be involved with energy regulation (Liu *et al*., 2011). Performing a metabolomics analysis is the logical next step for this system because using a metabolomics approach could give us a more global understanding of these organisms' metabolic interactions, and by relating these results to previous research we can gain a better understanding of this system as whole.

3.7 Resolving Species Through Metabolic Fingerprinting

Future research could also address the challenge of resolving different species and strains of bacteria within the genus *Acidithiobacllius*. The gold standard for bacterial identification is the use of 16s rRNA gene sequencing (Stackebrandt and Goebel, 1994) and it is not our intention to replace this method with metabolomic fingerprinting as the best tool for bacterial identification. However, the use of metabolic fingerprinting to

identify bacteria does have precedence in the literature in certain practical applications. In a clinical setting, metabolomic fingerprinting is used to identify different species of yeast in patients with yeast infections (reviewed in Bader, 2013). Yeast infections are caused by more than one species of yeast and are potentially deadly in patients that are immunocompromised. Different treatments are appropriate for different species, making identification crucial. In the past, 16s rRNA gene screening methods have been used, however the 16s rRNA method is time consuming and costly leading researchers to attempt to implement species identification through metabolomic fingerprinting. Currently, metabolomic analyses can correctly identify the species present 96% of the time, with improved metabolomics libraries this value will likely increase (Seng *et al*., 2009; Steensels *et al*., 2011). The research on the identification of the species of yeast causing yeast infections in a clinical setting establishes the feasibility for utilization of metabolomics fingerprinting to characterize microbial species. Following similar characterization protocols would allow us to ask fundamental questions about the amount of variation that exists within, and between, species and strains in the genus *Acidithiobacillus*. For example, an early experiment could involve characterizing the metabolome of five or more strains of bacteria currently assigned to the group *At. ferrooxidans* and comparing these, and the variation between them, to five strains currently assigned to the species *At. ferrivorans*. The results of said experiment would give us a better understanding of how much variation exists within a species belonging to either genus and if/to what degree we can actually separate and resolve different species and strains of bacteria belonging to these genera. In addition, globally there are many tailing ponds that have all been geographically isolated from each other, each with

isolated, and possibly different, species/strains of *Acidithiobacillus*. Currently it is thought that abiotic pressures such as pH, concentration of dissolved metals/oxygen as well as and total organic carbon as opposed to geographic location are the biggest predictors of microbial diversity present in tailings ponds (Kuang *et al*., 2013; Méndez-Garcıa *et al*., 2014; Mueller *et al*., 2011; Tan *et al*., 2009). Overall it isn't widely understood how/if these factors affect the role of *Acidithiobacillus* on a metabolic level. A large scale metabolomics analysis of the global diversity in the genus *Acidithiobacillus* could potentially lead us to better understanding the role of these bacteria in different abiotic conditions, as well as the amount of variation there is in that role.

If it is possible to resolve different strains of *Acidithiobacillus*, and ask question about adaptation to a variety of different conditions that are seen globally then it is also reasonable to assume we could ask some interesting questions about laboratory adaptation. Currently, there are a few strains of *At. ferrooxidans* that have had a large amount of research done on them, these strain are readily available for research and can be obtained easily from a place such as the American Type Culture Collection. Previous research done on *Drosophila melanogaster* shows that laboratory adapted strains of *D. melanogaster* behave and phenotypically vary in a significantly different way than wild caught strains (Orzoco-terWengel *et al*., 2012; Sgro and Partridge, 2000). The extent to which we can observe and characterize phenotypic differences between laboratory drosophila strains and wild caught drosophila strains begs the question do the strains of *At. ferrooxidans* available from the ATCC differ metabolically from a wild caught *At. ferrooxidans*. How do they differ and how much do they differ? Can laboratory adapted strains be used to just ask fundamental biological questions or can we still use these

strains to ask question related to the actual AMD environment? Based on previous studies of laboratory adaptation I would expect there to be a significant change in the metabolome in laboratory adapted *At. ferrooxidans* because of the changes that have been seen laboratory adapted *D. melanogaster*, and the change in *At. ferrooxidans* may be even more extreme because the generation time of bacteria is much smaller than that of drosophila.

Overall I think metabolomics is a good field of science to use to address both intra and inter species diversity present in the genus *Acidithiobacillus.* More commonly a genomics approach has been taken to ask questions about diversity and laboratory adaptation in bacteria. Although these questions can be asked using genomics there are some drawbacks with genomics techniques. Genomic information does not lead us directly to functional information, many of the potential genes identified in genomics analysis have no known function (Clare and King, 2003; Serres *et al*., 2001). This means that using genomics/genetics data we can determine if there has been a change in a species or strains of *Acidthiobacillus* but we have no idea what the functional result of that change is (Downes, 2004).

3.8 Concluding Summary

To summarize, we currently have developed a protocol to perform metabolomics in *At. ferrooxidans*, however as protocol development is an ongoing task there is still room to improve the protocol in order to gain a broader coverage of the chemical diversity present in the metabolome, quench the metabolism faster in order to ensure samples are representative, and detect metabolites that are present at low concentration due to low biomass. In addition, we can also begin to ask biological questions that are

relevant to *At. ferrooxidans* as well as the AMD environment. These biological questions would fall into two categories; community dynamics and metabolomic fingerprinting of a species. It is relevant to ask questions about community dynamics due to the relatively simple structure of the AMD community as defined by species richness. In addition, *At. ferrooxidans* is known to have a syntrophic relationship with members of the *Acidophilium* genus which results in a fivefold increase in the number of *At. ferrooxidans* cells present in a sample, obtaining a higher biomass will result in a higher concentration of metabolites thereby allowing us to ask biological questions as well as improving our methods ability to detect metabolites that are present at a low concentration. Finally, we will be able to ask questions about the metabolomic diversity present within genus *Acidithiobacillus* and also between two *Acidithiobacillus* species. Comparing the metabolomics fingerprint of a variety of strains belonging to the genus *Acidithiobacillus* will allow us to determine if we can resolve *At. ferrooxidans* using metabolomic fingerprinting. In addition, if we can resolve *At. ferrooxidans* we can ask fundamental about laboratory adaptation and diversity in *At. ferrooxidans*

References

Annesley, T.M. (2003). Ion suppression in mass spectrometry. *Clinical Chemistry 49(7)*, 1041–1044.

Auld, R.R., Myre, M., Mykytczuk, N.C.S., Leduc, L.G., and Merritt, T.J.S. (2013). Characterization of the microbial acid mine drainage microbial community using culturing and direct sequencing techniques. *Journal of Microbiological Methods 93(2)*, 108–115.

Bader, O. (2013). MALDI-TOF-MS-based species identification and typing approaches in medical mycology. *Proteomics*, *13(5)*, 788-799.

Baidoo, E. E., Benke, P. I., & Keasling, J. D. (2012). Mass spectrometry-based microbial metabolomics. *Microbial Systems Biology: Methods and Protocols*, 215-278.

Bajad, S.U., Lu, W., Kimball, E.H., Yuan, J., Peterson, C., and Rabinowitz, J.D. (2006). Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. *Journal of Chromatography A 1125(1*), 76–88.

Baker, B. J., & Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS microbiology ecology*, *44(2),* 139-152.

Bertrand, S., Schumpp, O., Bohni, N., Bujard, A., Azzollini, A., Monod, M., Gindro, K., & Wolfender, J. L. (2013). Detection of metabolite induction in fungal co-cultures on solid media by high-throughput differential ultra-high pressure liquid chromatography–time-of-flight mass spectrometry fingerprinting. *Journal of Chromatography A*, *1292*, 219-228.

Biers, E. J., Sun, S., & Howard, E. C. (2009). Prokaryotic genomes and diversity in surface ocean waters: interrogating the global ocean sampling metagenome. *Applied and environmental microbiology, 75(7),* 2221-2229.

Birkemeyer, C., Luedemann, A., Wagner, C., Erban, A., & Kopka, J. (2005). Metabolome analysis: the potential of in vivo labeling with stable isotopes for metabolite profiling. *Trends in biotechnology*, *23(1),* 28-33.

Bölling, C., & Fiehn, O. (2005). Metabolite profiling of Chlamydomonas reinhardtii under nutrient deprivation. *Plant physiology*, *139*(4), 1995-2005.

Bolten, C. J., & Wittmann, C. (2008). Appropriate sampling for intracellular amino acid analysis in five phylogenetically different yeasts. *Biotechnology letters*, *30*(11), 1993-2000.

Bond, P. L., Druschel, G. K., & Banfield, J. F. (2000). Comparison of acid mine drainage microbial communities in physically and geochemically distinct ecosystems. *Applied and Environmental Microbiology*, *66*(11), 4962-4971.

Buszewski, B., & Noga, S. (2012). Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique. *Analytical and bioanalytical chemistry*, *402*(1), 231-247.

Buziol, S., Bashir, I., Baumeister, A., Claaßen, W., Noisommit-Rizzi, N., Mailinger, W., & Reuss, M. (2002). New bioreactor-coupled rapid stopped-flow sampling technique for measurements of metabolite dynamics on a subsecond time scale. *Biotechnology and bioengineering*, *80*(6), 632-636.

Canelas, A. B., ten Pierick, A., Ras, C., Seifar, R. M., van Dam, J. C., van Gulik, W. M., & Heijnen, J. J. (2009). Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. *Analytical chemistry*, *81*(17), 7379-7389.

Chernushevich, I. V., Loboda, A. V., & Thomson, B. A. (2001). An introduction to quadrupole–time-of-flight mass spectrometry. *Journal of Mass Spectrometry*, *36*(8), 849-865.

Clare, A., & King, R. D. (2003). Predicting gene function in Saccharomyces cerevisiae. *Bioinformatics*, *19*(suppl 2), ii42-ii49.

Clark, W., & Christopher, K. (2000). An Introduction to DNA: Spectrophotometry, degradation, and the 'Frankengel'experiment. *Tested studies for laboratory teaching*, *22*, 81-99.

Dawson, P. H. (1986). Quadrupole mass analyzers: performance, design and some recent applications. *Mass Spectrometry Reviews, 5(1),* 1-37.

De Hoffmann, E. (2005). Mass Spectrometry. In Kirk-Othmer Encyclopedia of Chemical Technology, (5th Ed., Vol. 15, pp. 1–20). John Wiley & Sons, Inc.

de Koning, W., & van Dam, K. (1992). A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH. *Analytical biochemistry*, *204*(1), 118-123.

Dettmer, K., Aronov, P. A., & Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass spectrometry reviews, 26(1)*, 51-78.

Dopson, M., Baker-Austin, C., Hind, A., Bowman, J. P., & Bond, P. L. (2004). Characterization of Ferroplasma isolates and Ferroplasma acidarmanus sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments. *Applied and environmental microbiology*, *70*(4), 2079-2088.

Downes, S. M. (2004). Alternative splicing, the gene concept, and evolution. *History and philosophy of the life sciences*, 91-104.

Edwards, K. J., Gihring, T. M., & Banfield, J. F. (1999). Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment. *Applied and Environmental Microbiology*, *65*(8), 3627-3632.

El-Aneed, A., Cohen, A., & Banoub, J. (2009). Mass spectrometry, review of the basics: electrospray, MALDI, and commonly used mass analyzers. *Applied Spectroscopy Reviews, 44(3)*, 210-230.

Elhamili, A., Samuelsson, J., Bergquist, J., & Wetterhall, M. (2011). Optimizing the extraction, separation and quantification of tricyclic antidepressant drugs in human plasma with CE-ESI-TOF-MS using cationic-coated capillaries. *Electrophoresis*, *32*(6-7), 647-658.

Ershov, B. G. (1998). Radiation-chemical degradation of cellulose and other polysaccharides. *Russian Chemical Reviews*, *67*(4), 315-334.

Faijes, M., Mars, A. E., & Smid, E. J. (2007). Comparison of quenching and extraction methodologies for metabolome analysis of Lactobacillus plantarum. *Microbial cell factories*, *6*(1), 1.

Fiehn, O. (2001). Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comparative and functional genomics*, *2*(3), 155-168.

Gamache, P. H., Meyer, D. F., Granger, M. C., & Acworth, I. N. (2004). Metabolomic applications of electrochemistry/mass spectrometry. *Journal of the American Society for Mass Spectrometry*, *15*(12), 1717-1726.

Garcia, D. E., Baidoo, E. E., Benke, P. I., Pingitore, F., Tang, Y. J., Villa, S., & Keasling, J. D. (2008). Separation and mass spectrometry in microbial metabolomics. *Current opinion in microbiology*, *11*(3), 233-239.

Gonzalez, B., François, J., & Renaud, M. (1997). A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. *Yeast*, *13*(14), 1347-1355.

Grata, E., Boccard, J., Guillarme, D., Glauser, G., Carrupt, P. A., Farmer, E. E., Wolfender, J.L., & Rudaz, S. (2008). UPLC–TOF-MS for plant metabolomics: a sequential approach for wound marker analysis in Arabidopsis thaliana. *Journal of Chromatography B*, *871*(2), 261-270.

Grumbach, E. S., Wagrowski-Diehl, D. M., Mazzeo, J. R., Alden, B., & Iraneta, P. C. (2004). Hydrophilic interaction chromatography using silica columns for the retention of

polar analytes and enhanced ESI-MS sensitivity. *Lc Gc North America*, *22*(10), 1010-1023.

Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M., & Moritz, T. (2004). Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomic studies with gas chromatography/mass spectrometry. *Analytical biochemistry*, *331*(2), 283-295.

Gurung, A., & Chakraborty, R. (2009). The role of Acidithiobacillus ferrooxidans in alleviating the inhibitory effect of thiosulfate on the growth of acidophilic Acidiphilium species isolated from acid mine drainage samples from Garubathan, India. *Canadian journal of microbiology*, *55*(9), 1040-1048.

Harrison Jr, A. P. (1984). The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. *Annual Reviews in Microbiology*, *38*(1), 265-292.

Hein, J. E., & Blackmond, D. G. (2012). On the origin of single chirality of amino acids and sugars in biogenesis. *Accounts of chemical research*,*45*(12), 2045-2054.

Hoque, M. A., Ushiyama, H., Tomita, M., & Shimizu, K. (2005). Dynamic responses of the intracellular metabolite concentrations of the wild type and pykA mutant Escherichiacoli against pulse addition of glucose or NH 3 under those limiting continuous cultures. *Biochemical Engineering Journal*, *26*(1), 38-49.

Ismaiel, O. A., Halquist, M. S., Elmamly, M. Y., Shalaby, A., & Karnes, H. T. (2008). Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations. *Journal of Chromatography B, 875(2)*, 333-343.

Kamleh, M. A., Hobani, Y., Dow, J. A. T., & Watson, D. G. (2008). Metabolomic profiling of Drosophila using liquid chromatography Fourier transform mass spectrometry. *Febs Letters*, *582*(19), 2916-2922.

Kelly, D. P., & Wood, A. P. (2000). Reclassification of some species of Thiobacillus to the newly designated genera Acidithiobacillus gen. nov., Halothiobacillus gen. nov. and Thermithiobacillus gen. nov. *International Journal of Systematic and Evolutionary Microbiology*, *50*(2), 511-516.

Kim, S., Lee, D. Y., Wohlgemuth, G., Park, H. S., Fiehn, O., & Kim, K. H. (2013). Evaluation and optimization of metabolome sample preparation methods for Saccharomyces cerevisiae. *Analytical chemistry*, *85*(4), 2169-2176.

Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., & Fiehn, O. (2009). FiehnLib: mass spectral and retention index libraries for metabolomics based on

quadrupole and time-of-flight gas chromatography/mass spectrometry. *Analytical chemistry, 81(24)*, 10038-10048.

Klingenberg, M., & Pfaff, E. (1967). [104] Means of terminating reactions. *Methods in enzymology*, *10*, 680-684.

Knee, J. M., Rzezniczak, T. Z., Barsch, A., Guo, K. Z., & Merritt, T. J. (2013). A novel ion pairing LC/MS metabolomics protocol for study of a variety of biologically relevant polar metabolites. *Journal of Chromatography B*, *936*, 63-73.

Koek, M. M., Muilwijk, B., van der Werf, M. J., & Hankemeier, T. (2006). Microbial metabolomics with gas chromatography/mass spectrometry. *Analytical chemistry*, *78*(4), 1272-1281.

Koek, M. M., Jellema, R. H., van der Greef, J., Tas, A. C., & Hankemeier, T. (2011). Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives. *Metabolomics*, *7(3)*, 307-328.

Kuang, J. L., Huang, L. N., Chen, L. X., Hua, Z. S., Li, S. J., Hu, M., Li, J.T., & Shu, W. S. (2013). Contemporary environmental variation determines microbial diversity patterns in acid mine drainage. *The ISME journal*, *7*(5), 1038-1050.

Leduc, L. G., Trevors, J. T., & Ferroni, G. D. (1993). Thermal characterization of different isolates of Thiobacillus ferrooxidans. *FEMS microbiology letters*, *108*(2), 189-193.

Lee, D. Y., Bowen, B. P., & Northen, T. R. (2010). Mass spectrometry-based metabolomics, analysis of metabolite-protein interactions, and imaging. *Biotechniques*, *49*(2), 557-565.

Liu, H., Yin, H., Dai, Y., Dai, Z., Liu, Y., Li, Q., Jiang, H., & Liu, X. (2011). The co-culture of Acidithiobacillus ferrooxidans and Acidiphilium acidophilum enhances the growth, iron oxidation, and CO_2 fixation. *Archives of microbiology*, *193*(12), 857-866.

Luo, B., Groenke, K., Takors, R., Wandrey, C., & Oldiges, M. (2007). Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography–mass spectrometry. *Journal of chromatography A*, *1147*(2), 153-164.

Maharjan, R. P., & Ferenci, T. (2003). Global metabolite analysis: the influence of extraction methodology on metabolome profiles of Escherichia coli. *Analytical biochemistry*, *313*(1), 145-154.

Marchand, E. A., & Silverstein, J. (2003). The role of enhanced heterotrophic bacterial growth on iron oxidation by Acidithiobacillus ferrooxidans. *Geomicrobiology Journal*, *20*(3), 231-244.

Martínez, P., Gálvez, S., Ohtsuka, N., Budinich, M., Cortés, M. P., Serpell, C., Nakahigashi, K., Hirayama, A., Tomita, M., Soga, T., & Martínez, S. (2013). Metabolomic study of Chilean biomining bacteria Acidithiobacillus ferrooxidans strain Wenelen and Acidithiobacillus thiooxidans strain Licanantay. *Metabolomics*, *9*(1), 247-257.

MEND, 2005. Review of Disposal, Reprocessing and Reuse Options for Acidic Drainage Treatment Sludge. CANMET, Canada Centre for Mineral and Energy Technology.

Méndez-Garcıa, C., Mesa, V., Sprenger, R. R., Richter, M., Diez, M. S., Solano, J., Bargiela, R., Golyshina, O.V., Manteca, A., Ramos, J.L., & Gallego, J. R. (2014). Microbial stratification in low pH oxic and suboxic macroscopic growths along an acid mine drainage. The *ISME Journal*, *8*, 1259-1274.

Mohit, V., Archambault, P., & Lovejoy, C. (2015). Resilience and adjustments of surface sediment bacterial communities in an enclosed shallow coastal lagoon, Magdalen Islands, Gulf of St. Lawrence, Canada. *FEMS microbiology ecology*, *91*(5), fiv038.

Mueller, R. S., Dill, B. D., Pan, C., Belnap, C. P., Thomas, B. C., VerBerkmoes, N. C., Hettich, R.L., & Banfield, J. F. (2011). Proteome changes in the initial bacterial colonist during ecological succession in an acid mine drainage biofilm community. *Environmental microbiology*, *13*(8), 2279-2292.

Mushtaq, M. Y., Choi, Y. H., Verpoorte, R., & Wilson, E. G. (2014). Extraction for metabolomics: access to the metabolome. *Phytochemical Analysis*, *25*(4), 291-306.

Nelson, D. L., Lehninger, A. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry*. Macmillan.

Nicholson, J. K., & Lindon, J. C. (2008). Systems biology: metabonomics. *Nature*, *455* (7216), 1054-1056.

Olivares, J. A., & Houk, R. S. (1986). Suppression of analyte signal by various concomitant salts in inductively coupled plasma mass spectrometry. *Analytical Chemistry, 58(1),* 20-25.

Oliver, S. G., Winson, M. K., Kell, D. B., & Baganz, F. (1998). Systematic functional analysis of the yeast genome. *Trends in biotechnology*, *16*(9), 373-378.

Oldiges, M., Kunze, M., Degenring, D., Sprenger, G. A., & Takors, R. (2004). Stimulation, monitoring, and analysis of pathway dynamics by metabolic profiling in the aromatic amino acid pathway. *Biotechnology progress*, *20*(6), 1623-1633.

Orloff, J. (Ed.). (2008). *Handbook of charged particle optics*. CRC press.

Orozoco-terwengel, P. A. B. L. O., Kapun, M., Nolte, V., Kofler, R., Flatt, T., & Schloetterer, C. (2012). Adaptation of Drosophila to a novel laboratory environment reveals temporally heterogeneous trajectories of selected alleles. *Molecular ecology*, *21(20),* 4931-4941.

Paczia, N., Nilgen, A., Lehmann, T., Gätgens, J., Wiechert, W., & Noack, S. (2012). Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. *Microbial cell factories*, *11*(1), 1.

Pan, Z., & Raftery, D. (2007). Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and bioanalytical chemistry*, *387*(2), 525-527.

Pardue, H. L. (1997). Counterpoint The inseparable triad: analytical sensitivity, measurement uncertainty, and quantitative resolution. *Clinical chemistry, 43(10),* 1831-1837.

Rabinowitz, J. D., & Kimball, E. (2007). Acidic acetonitrile for cellular metabolome extraction from Escherichia coli. *Analytical Chemistry*, *79*(16), 6167-6173.

Schaefer, U., Boos, W., Takors, R., & Weuster-Botz, D. (1999). Automated sampling device for monitoring intracellular metabolite dynamics. *Analytical biochemistry*, *270*(1), 88-96.

Schauer, A. J., Lott, M. J., Cook, C. S., & Ehleringer, J. R. (2005). An automated system for stable isotope and concentration analyses of CO2 from small atmospheric samples. *Rapid Communications in Mass Spectrometry*,*19*(3), 359-362.

Schmidt, C. W. (2004). Metabolomics: what's happening downstream of DNA. *Environmental Health Perspectives, 112(7)*, A410.

Schrenk, M. O., Edwards, K. J., Goodman, R. M., Hamers, R. J., & Banfield, J. F. (1998). Distribution of Thiobacillus ferrooxidans and Leptospirillum ferrooxidans: implications for generation of acid mine drainage. *Science*, *279*(5356), 1519-1522.

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P. E., Rolain, J. M., & Raoult, D. (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases*, *49*(4), 543-551.

Serres, M. H., Gopal, S., Nahum, L. A., Liang, P., Gaasterland, T., & Riley, M. (2001). A functional update of the Escherichia coli K-12 genome. *Genome biology*, *2*(9), 1.

Sgro, C. M., & Partridge, L. (2000). Evolutionary responses of the life history of wild-caught Drosophila melanogaster to two standard methods of laboratory culture. *The American Naturalist*, *156*(4), 341-353.

Singer, P. C., & Stumm, W. (1970). Acidic mine drainage: the rate-determining step. *Science*, *167*(3921), 1121-1123.

Smart, K. F., Aggio, R. B., Van Houtte, J. R., & Villas-Bôas, S. G. (2010). Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. *Nature Protocols*, *5*(10), 1709-1729.

S.G. Villas-Boˆas, U. Roessner, M.A.E. Hansen, J. Smedsgaard, J. Nielse (Eds.), Sampling and sample preparation Metabolome Analysis: An Introduction, Wiley–Interscience, Hoboken, NJ, 2007, pp. 39–82

Shin, M. H., Lee, D. Y., Liu, K. H., Fiehn, O., & Kim, K. H. (2010). Evaluation of sampling and extraction methodologies for the global metabolic profiling of Saccharophagus degradans. *Analytical chemistry*, *82*(15), 6660-6666.

Smilde, A. K., van der Werf, M. J., Bijlsma, S., van der Werff-van der Vat, B. J., & Jellema, R. H. (2005). Fusion of mass spectrometry-based metabolomics data. *Analytical chemistry*, *77*(20), 6729-6736.

Spura, J., Reimer, L. C., Wieloch, P., Schreiber, K., Buchinger, S., & Schomburg, D. (2009). A method for enzyme quenching in microbial metabolome analysis successfully applied to Gram-positive and Gram-negative bacteria and yeast. *Analytical biochemistry*, *394*(2), 192-201.

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, *44*(4), 846-849.

Streten-Joyce, C., Manning, J., Gibb, K. S., Neilan, B. A., & Parry, D. L. (2013). The chemical composition and bacteria communities in acid and metalliferous drainage from the wet–dry tropics are dependent on season. *Science of the Total Environment*, *443*, 65-79.

Tan, G. L., Shu, W. S., Zhou, W. H., Li, X. L., Lan, C. Y., & Huang, L. N. (2009). Seasonal and spatial variations in microbial community structure and diversity in the acid stream draining across an ongoing surface mining site. *FEMS microbiology ecology*, *70*(2), 277-285.

Taylor, P. J. (2005). Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical biochemistry, 38(4),* 328-334.

Temple, K. L., & Colmer, A. R. (1951). The autotrophic oxidation of iron by a new bacterium: Thiobacillus ferrooxidans. *Journal of Bacteriology*, *62*(5), 605.

Tillack, J., Paczia, N., Nöh, K., Wiechert, W., & Noack, S. (2012). Error propagation analysis for quantitative intracellular metabolomics. *Metabolites*,*2*(4), 1012-1030.

Tiller, P. R., Yu, S., Castro-Perez, J., Fillgrove, K. L., & Baillie, T. A. (2008). High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line'approach for metabolite identification studies. *Rapid Communications in Mass Spectrometry*, *22*(7), 1053-1061.

t'Kindt, R., Scheltema, R. A., Jankevics, A., Brunker, K., Rijal, S., Dujardin, J. C., Breitling, R., Watson, D.G., Coombs, G.H., & Decuypere, S. (2010). Metabolomics to unveil and understand phenotypic diversity between pathogen populations. *PLoS Neglected Tropial Diseases*, *4*(11), e904.

Tweeddale, H., Notley-McRobb, L., & Ferenci, T. (1998). Effect of slow growth on metabolism of Escherichia coli, as revealed by global metabolite pool ("metabolome") analysis. *Journal of bacteriology*, *180*(19), 5109-5116.

van der Werf, M. J., Overkamp, K. M., Muilwijk, B., Coulier, L., & Hankemeier, T. (2007). Microbial metabolomics: toward a platform with full metabolome coverage. *Analytical biochemistry*, *370*(1), 17-25.

Verhoeven, H. A., de Vos, C. R., Bino, R. J., & Hall, R. D. (2006). Plant metabolomics strategies based upon quadrupole time of flight mass spectrometry (QTOF-MS). In *Plant Metabolomics* (pp. 33-48). Springer Berlin Heidelberg.

Villas-Bôas, S. G., & Bruheim, P. (2007). Cold glycerol–saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells. *Analytical biochemistry*, *370*(1), 87-97.

Visser, D., van Zuylen, G. A., van Dam, J. C., Oudshoorn, A., Eman, M. R., Ras, C., van Gulik, W.M., Frank, J., van Dedem, G.W., & Heijnen, J. J. (2002). Rapid sampling for analysis of in vivo kinetics using the BioScope: a system for continuous-pulse experiments. *Biotechnology and Bioengineering*, *79*(6), 674-681.

Wan, Q. H., Davies, M. C., Shaw, P. N., & Barrett, D. A. (1996). Retention behavior of ionizable isomers in reversed-phase liquid chromatography: a comparative study of porous graphitic carbon and octadecyl bonded silica. *Analytical chemistry*, *68*(3), 437-446.

Wang, X., Xie, Y., Gao, P., Zhang, S., Tan, H., Yang, F., Lian, R., Tian, J. , & Xu, G. (2014). A metabolomics-based method for studying the effect of yfcC gene in Escherichia coli on metabolism. *Analytical biochemistry*, *451*, 48-55.

Waksman, S. A., & Joffe, J. S. (1922). Microörganisms Concerned in the Oxidation of Sulfur in the Soil: II. Thiobacillus Thiooxidans, a New Sulfur-oxidizing Organism Isolated from the Soil. *Journal of Bacteriology*, *7*(2), 239–256.

Water, 2013. Stepwave: Enhancing sensitivity and Robustness. Waters Literature No 720004157EN

Wilm, M. (2011). Principles of Electrospray Ionization. Mol. Cell. Proteomics 10, 1–8

Winder, C. L., Dunn, W. B., Schuler, S., Broadhurst, D., Jarvis, R., Stephens, G. M., & Goodacre, R. (2008). Global metabolic profiling of Escherichia coli cultures: an evaluation of methods for quenching and extraction of intracellular metabolites. *Analytical Chemistry*, *80*(8), 2939-2948.

Wishart, D. S. (2008). Quantitative metabolomics using NMR. *Trends in Analytical Chemistry*, *27*(3), 228-237.

Wittmann, C., Krömer, J. O., Kiefer, P., Binz, T., & Heinzle, E. (2004). Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria. *Analytical biochemistry*, *327*(1), 135-139.

Wu, T., Zivanovic, S., Hayes, D. G., & Weiss, J. (2008). Efficient reduction of chitosan molecular weight by high-intensity ultrasound: Underlying mechanism and effect of process parameters. *Journal of Agriculture and Food Chemistry, 56*, 5112–5119.

Xia, J., Psychogios, N., Young, N., and Wishart, D.S. (2009). MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Residues. 37, W652–W660.

Xia, J., Mandal, R., Sinelnikov, I.V., Broadhurst, D., and Wishart, D.S. (2012). MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis. Nucleic Acids Residues.

Xu, Y. J., Wang, C., Ho, W. E., & Ong, C. N. (2014). Recent developments and applications of metabolomics in microbiological investigations. *Trends in Analytical Chemistry*, *56*, 37-48.

Yanes, O., Tautenhahn, R., Patti, G. J., & Siuzdak, G. (2011). Expanding coverage of the metabolome for global metabolite profiling. *Analytical chemistry*, *83*(6), 2152-2161.

Zaldivar, J., Borges, A., Johansson, B., Smits, H., Villas-Bôas, S., Nielsen, J., & Olsson, L. (2002). Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting Saccharomyces cerevisiae. *Applied microbiology and biotechnology*, *59*(4-5), 436-442.

Zivkovic, A. M., Wiest, M. M., Nguyen, U. T., Davis, R., Watkins, S. M., & German, J. B. (2009). Effects of sample handling and storage on quantitative lipid analysis in human serum. *Metabolomics*, *5*(4), 507-516.

Appendices

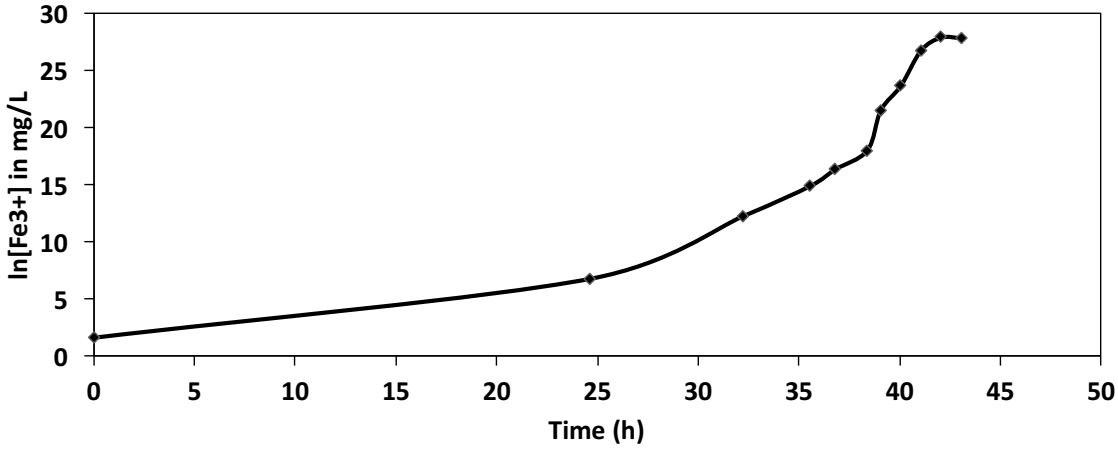

Figure A1: The natural logarithm of the ferric iron concentrations versus time as determined by spectrophometic analysis of iron production when *At. ferrooxidans* is grown in TK media. The assumption being that the exponential phase of iron production matches the exponential phase of *At. ferrrooxidans* growth.

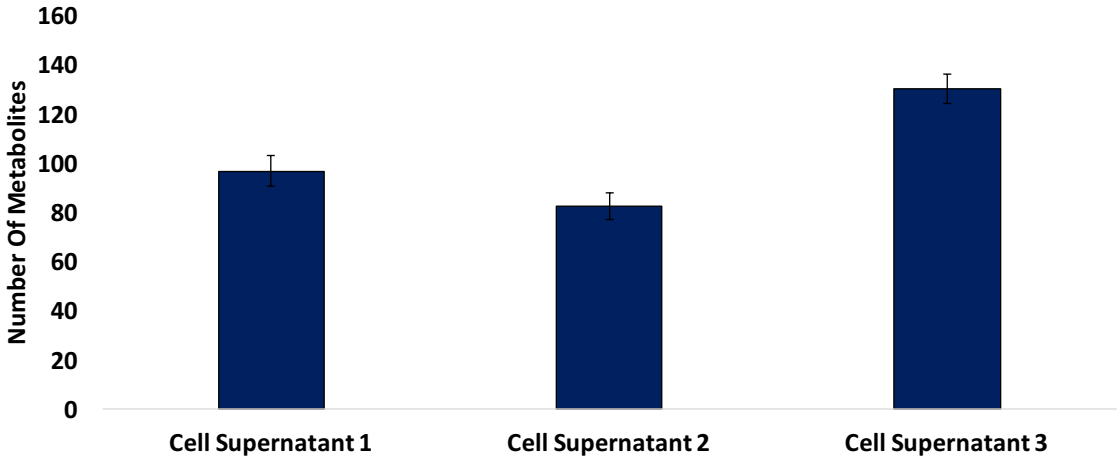

Figure A2: Mean of technical replicates(n=3) of the cell supernatant.

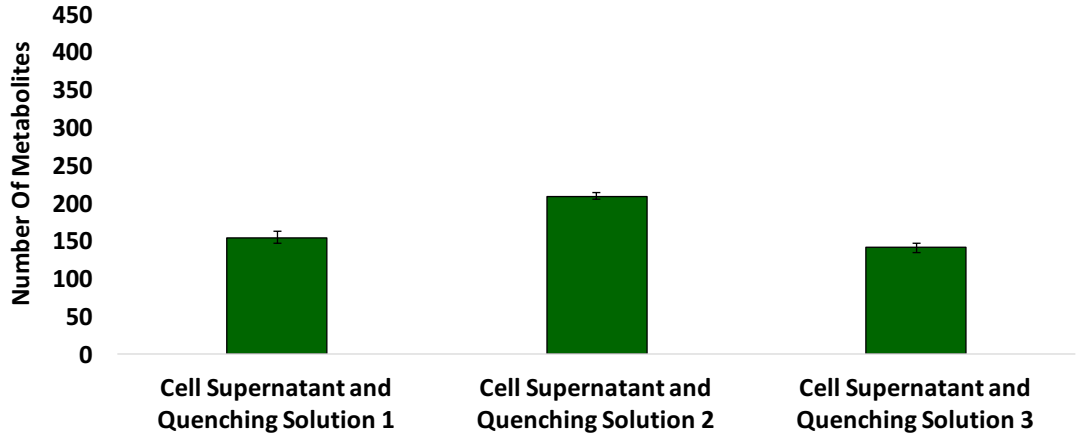

Figure A3: Mean of technical replicates(n=3) of cell supernatant and ammonium formate quenching solution.

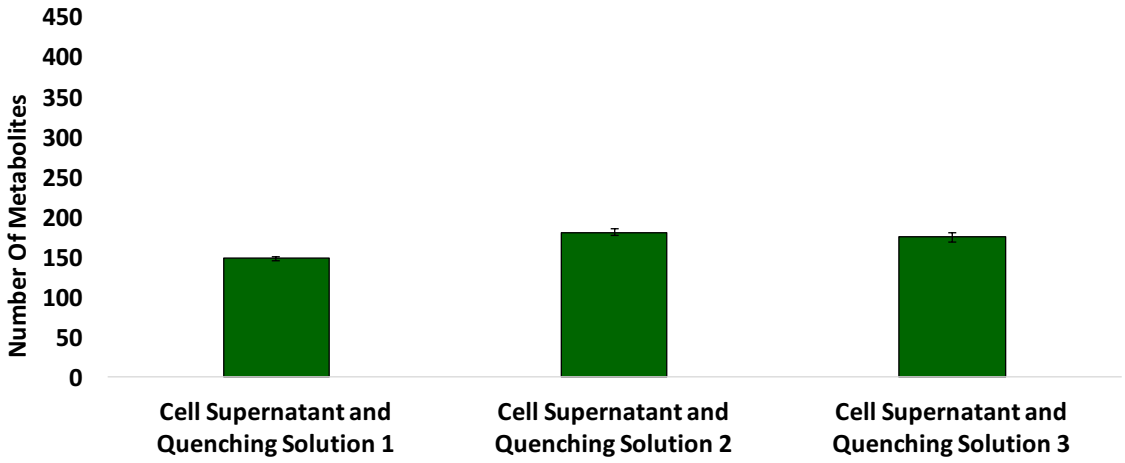

Figure A4: Mean of the technical replicates(n=3) of cell supernatant and TK quenching solution.

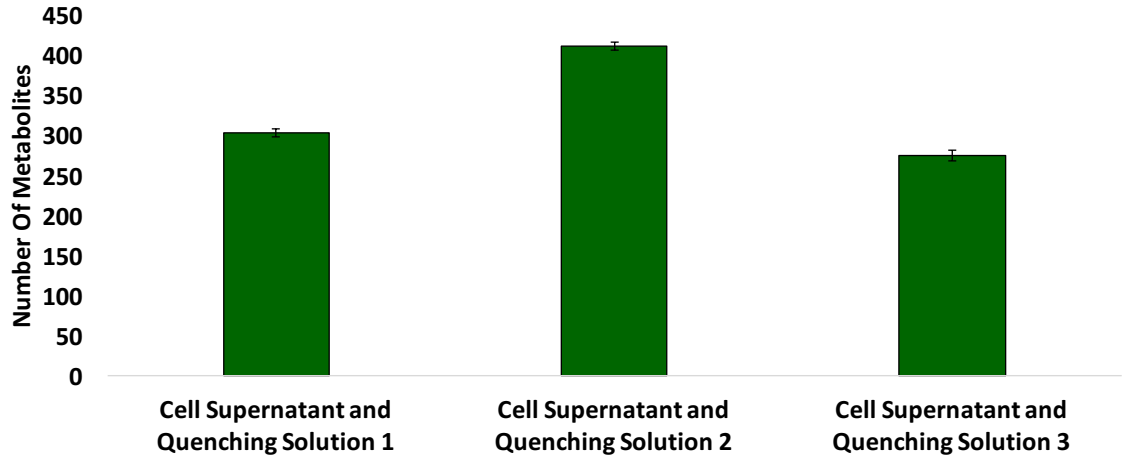

Figure A5: Mean of technical replicates(n=3) of cell supernatant and physiological saline quenching solution.

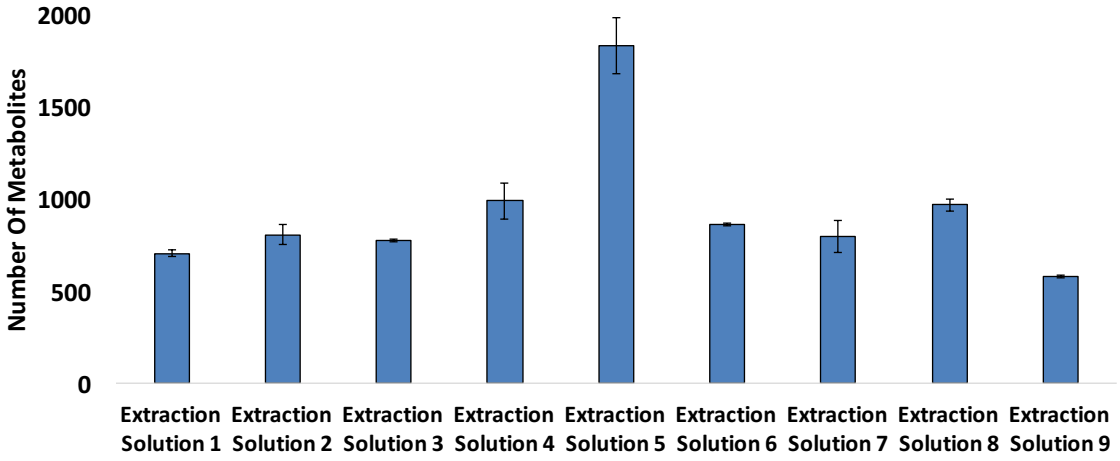

Figure A6: Mean of technical replicates(n=3) of the isopropanol:methanol:water extraction.

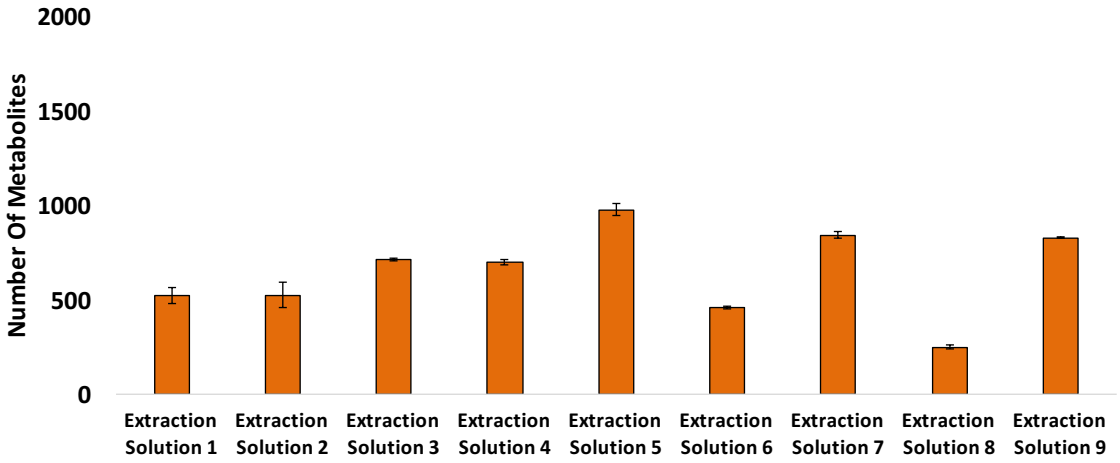

Figure A7: Mean of technical replicates(n=3) of the chloroform:methanol:water extraction.

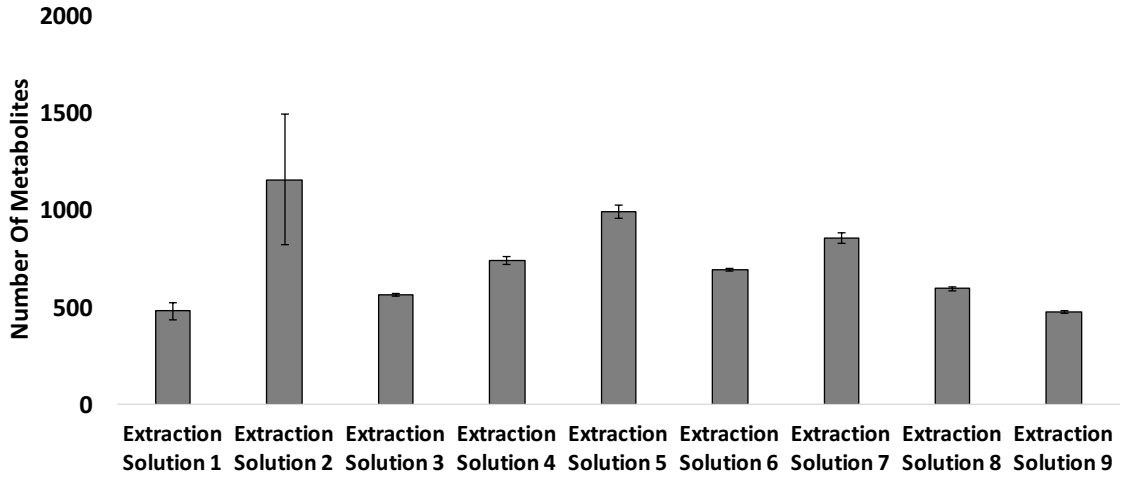

Figure A8: Mean of technical replicates(n=3) of the acidic acetonitrile: water extraction.

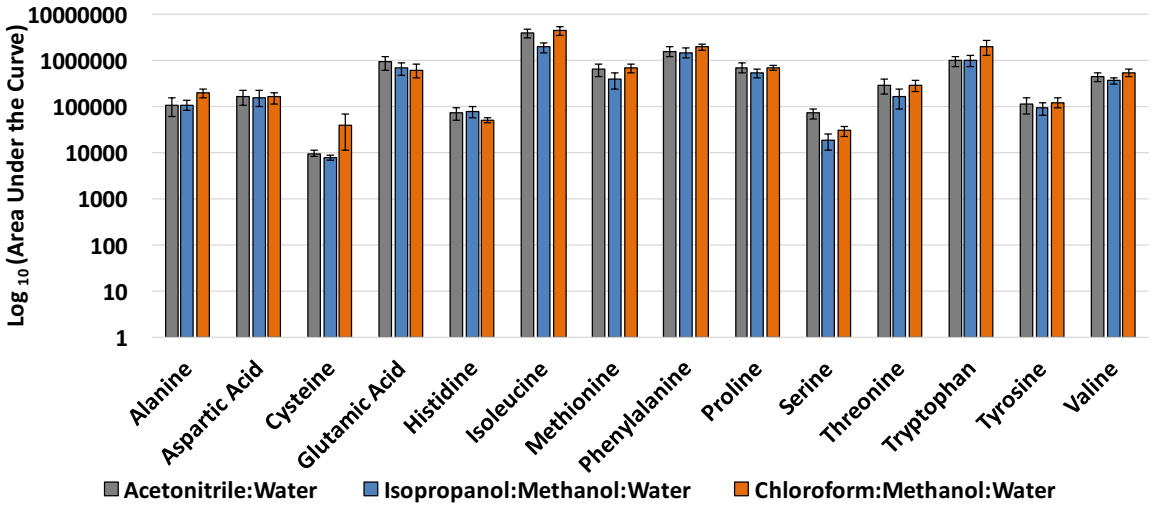

Figure A9: Mean of technical variation(n=3) in fourteen ^{13}C labelled standards in three extraction solutions.